EXERCISE AND OXIDATIVE STRESS: IMPLICATIONS IN HEALTH AND DISEASE

by

Gareth William Davison B.A (Hons) M.Sc.

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ABSTRACT

This thesis presents studies investigating the effects of exercise on free radical production as measured by *ex vivo* Electron Spin Resonance (ESR) spectroscopy and the by-products of lipid peroxidation.

It has been proposed that exercising in hypoxia may increase free radical production. Thus study one investigated the effects of aerobic exercise performed in normobaric hypoxia (F_IO_2 = 16%) on free radical production. Results demonstrate that hypoxic exercise does not increase (*time x group*, $P > 0.05$) systemic free radical levels. However, exercise performed at 55% $\dot{V}O_{2peak}$ markedly increased (*rest vs. exercise*, $P < 0.05$) the concentration of free radical species and lipid hydroperoxides (LH) in systemic blood. This increase was related to an exercise-induced increase (*rest vs. exercise*, $P < 0.05$) in oxygen consumption, implicating the mitochondria as a potential source of free radicals.

Type 1 diabetes is a disorder characterised by compromised antioxidant defences and increased oxidative stress. Study two investigated the effects of exhaustive exercise on these parameters in type 1 diabetic patients. No selective exercise difference (*time x group*, $P > 0.05$) was observed in free radical production between groups, although, diabetic patients had a higher systemic concentration of free radicals and LH (*diabetic vs. control*, $P < 0.05$). In addition, exhaustive exercise increased overall free radical and LH concentration (*rest vs. exercise*, $P < 0.05$). These changes may be related to glucose auto-oxidation and mitochondrial electron '*leakage*' as potential sources of increased free radical production.

Study three determined the effects of exercise and ascorbic acid supplementation on free radical production in these patients. Ascorbic acid supplementation did not selectively decrease free radical production in type 1 diabetic patients (*group x treatment*, $P > 0.05$), however, ascorbic acid supplementation decreased overall oxidative stress levels (*ascorbic acid vs. placebo*, $P < 0.05$) and free radicals post-exercise (*time x treatment*, $P < 0.05$). This research demonstrates that ascorbic acid is an effective antioxidant in decreasing oxidative stress in human blood.

A series of *in vitro* studies were performed in order to attempt to identify the origin of the free radical species. These results suggest that the free radicals are oxygen-centred and derived from the oxidation of phospholipid membranes. This work demonstrates that (1) physical exercise *per se* can increase oxygen-centred free radical production, (2) type 1 diabetic patients are more susceptible to oxidative stress, and (3) ascorbic acid is effective in attenuating oxidative stress levels in humans.

CERTIFICATE OF RESEARCH

This is to certify that, except where specific reference is made, the work described in this thesis is the result of the candidate. Neither this thesis, nor any part of it, has been presented, or is currently submitted, in candidature for any degree at any other University.

Signed: .....

Gareth W. Davison *(Candidate)*

Signed: .....

Professor Bruce Davies *(Director of Studies)*

Date: 22/7/02

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GLOSSARY OF NOMENCLATURE AND ABBREVIATIONS

NOMENCLATURE

Reactive Oxygen Species (ROS) – Collective term for oxygen-derived molecular species

Reactive Nitrogen Species (RNS) – Collective term for nitrogen-derived molecular species

Free Radical – A chemical species in which one or more unpaired electrons occupy an outer orbital.

Radical – Synonymous with the term free radical.

Electron Paramagnetic Resonance (EPR) – Analytical technique used in the direct measurement of free radical species.

Electron Spin Resonance (ESR) – Synonymous with the term electron paramagnetic resonance and used interchangeably.

Oxidative Stress – Collective name for detrimental free radical reactions.

Antioxidant – Chemical substance which has the ability to prevent and scavenge reactive oxygen species.

Lipid peroxidation – Process whereby polyunsaturated fatty acid molecules are subjected to attack and degradation by reactive oxygen species.

Maximal Oxygen Uptake ($\dot{V}O_{2\,max}$) – Maximum rate at which an individual can take up and utilise oxygen whilst breathing at sea level.

Oxygen Uptake ($\dot{V}O_2$) – Volume of oxygen consumed per unit of time.

Heart Rate (HR) – Frequency with which the heart pumps blood around the body.

ABBREVIATIONS

$a\beta_H$ – Hydrogen coupling constant

AMP, ADP and ATP – Adenosine 5' – mono – di – and tri – phosphate

a_N – Nitrogen coupling constant

ANOVA – Analysis of variance

CAT – Catalase

CCO – Cytochrome *c* oxidase

cm – Centimetre

CO_2 – Carbon dioxide

Cu – Copper

CuZnSOD – Copper-zinc superoxide dismutase

EDTA – Ethylene-diamine-tetra-acetate

EPR – Electron paramagnetic resonance spectroscopy (Arbitrary units)

ESR – Electron spin resonance spectroscopy (Arbitrary units)

Fe – Iron

g – gauss

Ghz – Gigahertz

GPX – Glutathione peroxidase

GR – Glutathione reductase

GSH – Glutathione

GSSG – Glutathione disulfide (oxidised)

GST – Glutathione sulfur-transferase

HbA_{1C} – Glycosylated haemoglobin (%)

Hb – Haemoglobin concentration (g/dl)

Hct – Haematocrit (%)

HOCl – Hydrochlorous acid

$HOO^{\cdot}$ – Hydroperoxyl radical

H_2O – Water

H_2O_2 – Hydrogen peroxide

HPLC – High performance liquid chromatography

kg – Kilograms

L – Litre

L· – Lipid radical

LH – Lipid hydroperoxide ($\mu mol \cdot L^{-1}$)

LOOH – Lipid peroxides

M – Molar

MDA – Malondialdehyde ($\mu mol \cdot L^{-1}$)

mg – Milligram

ml – Millilitre

mM – Millimolar

mmol.L – Millimoles per litre

Mn – Manganase

MnSOD – Manganese superoxide dismutase

mT – Modulation amplitude

mW – Microwave power

n – Sample number

NAD – Nicotinamide adenine dinucleotide

NADH – Nicotinamide adenine dinucleotide (reduced)

NADP – Nicotinamide adenine dinucleotide phosphate

NADPH – Nicotinamide adenine dinucleotide phosphate (reduced)

$NO^{\cdot}$ – Nitric oxide

O_2 – Molecular oxygen

$O_2^{\cdot -}$ - Superoxide anion

$^1O_2^{\cdot}$ – Singlet oxygen

% - Percentage

^{O}C – Centigrade

OH $^{\cdot}$ – Hydroxyl radical

ONOO $^{-}$ - Peroxynitrite

P – Level of significance (Probability)

PBN – α-phenyl-*tert*-butylnitrone

PCV – Packed cell volume (L/L)

PUFA – Polyunsaturated fatty acid

PV – Plasma volume (ml)

R $^{\cdot}$ – Alkyl radical

RO $^{\cdot}$ – Alkoxyl radical

ROO $^{\cdot}$ – Peroxyl radical

RPE – Rate of perceived exertion

SD – Standard deviation

Sec(s) – Second(s)

SOD – Superoxide dismutase

SST – Serum separation tubes

TBA – Thiobarbituric acid

TBARS – Thiobarbituric acid reactive substances ($\mu mol \cdot L^{-1}$)

μL – Microlitre

wt – Weight (kg)

XO – Xanthine oxidase

Zn – Zinc

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RESEARCH COMMUNICATIONS ASSOCIATED WITH THIS THESIS

Peer reviewed scientific presentations:

Davison, G.W, George, L, Jackson, S.K, Davies, B, Young, I.S, Peters, J.R, Bailey, D.M, Ashton, T. (2000) *Application of EPR Spectroscopy in Assessing Free Radical Concentration Following Exhaustive Exercise in Humans.* Presented to the Society for Free Radical Research *(Europe)*, Liverpool, 20th-22nd July 2000.

Davison, G.W, George, L, Jackson, S.K, Davies, B, Young, I.S, Peters, J.R, Bailey, D.M, Ashton, T. (2000) *Application of Electron Spin Resonance Spectroscopy to Determine the Pro-oxidant Effects of Physical Exercise in Man.* Presented to the British Association of Sport and Exercise Sciences, Liverpool, 28th-2nd October 2001.

Davison, G.W, George, L, Jackson, S.K, Davies, B, Young, I.S, Peters, J.R, Bailey, D.M, Ashton, T. (2001) *Evidence for Increased Oxidant Production in Insulin-Dependent Diabetic Patients: An EPR Spectroscopic Study.* Presented to Diabetes UK Annual Professional Conference, Glasgow, 4th-6th April 2001.

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Bailey, D.M, Davies, B, ***Davison, G.W,*** Young, I.S. (2000) *Free Radical Damage at High Altitude: Isolating the Source and Implications for the Pathophysiology of Acute Mountain Sickness.* News. Int. Soc. Mount. Med. 10: 3-13.

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Davison, G.W, George, L, Jackson, S.K, Davies, B, Young, I.S, Peters, J.R, Bailey, D.M, Ashton, T. (2001) *Direct and Indirect Metabolic Evidence for Free Radical Generation in Type 1 Diabetes.* Diabetes. 50: (2) 483.

Davison, G.W, George, L, Jackson, S.K, Davies, B, Young, I.S, Peters, J.R, Bailey, D.M, Ashton, T. (2000) *Application of EPR Spectroscopy in Assessing Free Radical Concentration Following Exhaustive Exercise in Humans.* Proceedings of the Society for Free Radical Research (Europe) 55.

Manuscripts under preparation

Davison, G.W, George, L, Jackson, S.K, Davies, B, Young, I.S, Peters, J.R, Bailey, D.M, Ashton, T. *Exercise, Free Radicals and Lipid Peroxidation in Type 1 Diabetes Mellitus: An ESR Spin Trapping Study* (Journal of Applied Physiology).

Davison, G.W, George, L, Jackson, S.K, Davies, B, Young, I.S, Peters, J.R, Bailey, D.M, Ashton, T. *Free Radical Production in Young Type 1 Diabetes Mellitus: The Effect of Exercise and Ascorbic Acid Supplementation.* (Diabetes Care).

Davison, G.W, Bailey, D.M, Castell, L, Morgan, R.M, Hiscock, N, Young, I.S, Boisseau, N, Garcia, J.M, Grace, F, Ashton, T, Davies, B. *The Effect of Aerobic Exercise and Normobaric Hypoxia on Free Radicals and Selected Indices of Lipid Peroxidation in Humans.* (Medicine and Science in Sport and Exercise).

Davison, G.W, Garcia, J.M, Morgan, R.M, Young, I.S, Bailey, D.M, Ashton, T, Davies, B. *Biological Variation of Free Radicals, Lipid Peroxidation and Selected Antioxidant Indices in Man.* (Clinical Biochemistry).

Chapter 1

General Introduction

1.0 – Introduction

Free radical reactions are recognised as important mechanisms involved in potentially destructive biological processes (Gopaul, 1997). However to date, the majority of the literature has focused on measuring free radical-mediated lipid peroxidation as opposed to the free radicals themselves. Lipid peroxidation is now implicated in a wide range of situations, including modification of membrane structure associated with physical exercise (Alessio and Cutler, 1990) and a number of diseases such as atherosclerosis (Halliwell, 2000), Crohns disease (Wendland *et al,* 2001), and diabetes mellitus (Griesmacher *et al*, 1995). Defining the importance of lipid peroxidation in physical exercise and pathology can be resolved to a large extent by a better understanding of the events involved at key stages of the peroxidative process (Gopaul, 1997). Lipid peroxidation is known to produce secondary and tertiary free radical species which may damage adjacent lipid molecules (Stolze *et al*, 2000), however due to their short half-life and low steady state concentrations their direct measurement in biological samples is particularly difficult (Davies and Timmins, 1996).

Electron Spin Resonance (ESR) spectroscopy has the ability to detect paramagnetic free radical species of low concentration in biological and biochemical systems without destroying or modifying the substance in question (Ingram, 1969). It is arguably the most sensitive, specific and direct method of measuring free radical species (Davies and Timmins, 1996), but is currently under-utilised in the exercise and clinical setting.

Although physical exercise is largely regarded as beneficial to human health (Bouchard, 2000), evidence suggests that exercise may result in elevated free radical production which may elicit potentially toxic pertubations in cellular homeostasis (Alessio 1993, Ashton *et al* 1999). This has recently been confirmed in exhaustive exercise using ESR spectroscopy and lipid peroxidation (Ashton *et al*, 1998). There are however no published ESR data demonstrating the production of free radical species by prolonged aerobic exercise performed in normobaric hypoxia. As previous research has shown that exercise in hypoxia can elevate the by-products of lipid peroxidation (Bailey, 2001), it is hypothesised that exercise performed in hypoxia may increase the concentration of systemic free radical species. Thus application of ESR to the measurement of free radicals in the venous circulation would provide new knowledge

regarding oxidative stress and exercise performed in a reduced partial pressure of oxygen.

Research has also suggested that patients with diabetes mellitus are susceptible to increased levels of oxidative stress (Santini *et al* 1997, Griesmacher *et al* 1995), and recently this notion has been combined with exercise to show that patients with type 1 diabetes are exposed to elevated lipid peroxidation levels (Laaksonen *et al*, 1996). Within the literature there appears to be no published studies that have used ESR spectroscopy with or without exercise to examine the free radical concentration in type 1 diabetes. It is thus hypothesised that increased susceptibly to oxidative stress at rest or following exhaustive exercise in type 1 diabetes may be measured by ESR spectroscopy. The application of ESR spectroscopy to these patients would provide novel data, and may provide the basis for a more routine measurement of oxidative stress in clinical medicine.

Antioxidant supplementation has proven to be an effective strategy in combating oxidative stress (Ashton *et al*, 1999). It is suggested that ascorbic acid supplementation may decrease oxidative stress levels in diabetes. To this authors knowledge, there are no published data using ESR spectroscopy to ascertain the effect of ascorbic acid supplementation on resting or exercise-induced free radical production in type 1 diabetes mellitus patients. This approach may contribute towards making a recommendation for larger scale trials on the therapeutic use of antioxidant supplementation in type 1 diabetes mellitus.

This thesis will endeavour to examine the link between oxidants and antioxidants in health and disease. More specifically, the innovative technique of ESR spectroscopy in conjunction with spin trapping will assess directly the concentration of free radical species in blood of healthy and diabetic subjects before and following exercise. Measures of lipid peroxidation will be used as supporting markers of oxidative stress. The effect of ascorbic acid supplementation on the ESR signal intensity in diabetic patients will also be examined.

1.1 - Experimental aims and objectives

The principal aim of this work is to utilise ESR spectroscopy in conjunction with spin trapping to detect free radical species in the venous blood of apparently healthy and patient volunteers before and following exercise.

The primary objectives may be outlined as follows:

Pilot Studies - (a) To establish biological variation and critical difference of PBN adduct, measures of lipid peroxidation and individual antioxidants in human blood.

Study 1 - (a) To validate ESR spectroscopy as a direct measure of free radical production in exercise of 2 hr duration by comparing signal changes with conventional lipid peroxidation assays.

(b) To measure and quantify the degree of free radical mediated oxidative stress and antioxidant status in acute normobaric hypoxic exercise.

Study 2 - (a) To validate ESR spectroscopy as a direct measure of free radical production in patients with type 1 diabetes mellitus by comparing signal changes with conventional lipid peroxidation assays.

(b) To measure and quantify the degree of free radical mediated oxidative stress in patients with type 1 diabetes mellitus in comparison to matched healthy control subjects.

(c) To quantify plasma antioxidant status in patients with type 1 diabetes mellitus and to comment on its role in oxidative stress in these patients.

(d) To correlate resting and exercise-induced changes in free radical production in type 1 diabetes mellitus patients with ambient and long-term measures of glycaemic control (%HbA_{1c}) and $\dot{V}O_{2\,max}$.

Study 3 – (a) To examine the effect of ascorbic acid supplementation on oxidative stress parameters in type 1 diabetes before and following exercise.

In vitro studies – (a) To attempt to establish whether the free radicals detected in the main studies originate from polyunsaturated fatty acids (PUFA) and whether the free radicals detected are solely from one particular PUFA or a combination of PUFA.

1.2 – Overview of thesis

This thesis is presented in nine main chapters, and are briefly outlined in the following paragraphs:

Chapter 2:

The review of literature will examine existing knowledge pertaining to free radical mechanisms and exercise-induced oxidative stress/damage *in vivo*. An overview of antioxidant biochemistry and the pathophysiology of diabetes mellitus and its relation to free radicals will also be provided.

Chapter 3:

General methodology will open with a brief overview of ESR theory. A description of the medical, physiological and statistical testing procedures, in addition to biochemical analysis will be given. This chapter will conclude with various preliminary quality control experiments conducted.

Chapter 4:

The pilot studies were designed to investigate the analytical and biological variation in determining the critical difference for free radicals, lipid peroxidation by-products and selected antioxidant vitamins.

Chapter 5:

Study one was designed to investigate the relationship between free radical-mediated oxidative stress and acute physical exercise in normobaric hypoxia.

Chapter 6:

Study two was designed to investigate the relationship between free radical-mediated oxidative stress, type 1 diabetes mellitus and healthy control subjects.

Chapter 7:

This intervention and final human study was designed to investigate the effects of ascorbic acid supplementation on free radical-mediated oxidative stress, type 1 diabetes mellitus and healthy control subjects.

Chapter 8:

This chapter will present four *in vitro* studies attempting to elucidate the site of origin and identity of the free radical species detected by ESR spectroscopy in the human studies.

Chapter 9:

The synthesis of findings will include the testing of the null hypothesis and the general discussion. This chapter will integrate and summarise the research findings of all studies and consider the physiological and biochemical implications of exercise and oxidative stress in health and disease.

Chapter 2

Review of Literature

2.0 - Introduction to area of interest

Free radical research is a contemporary feature of biomedical journals and international conferences, with a plethora of exciting new information relating to health and disease discussed and presented. Free radical metabolism is implicated in over 100 human disease states, ranging from diabetes and rheumatoid arthritis to cystic fibrosis, to name but a few. In 1984, Halliwell and Gutteridge claimed that this wide range of disorders implies that oxidative stress is not something obscure, but that their increased formation is associated with tissue injury in most, if not all, human diseases. It is not known whether oxidative stress contributes towards the onset of a disease process or is merely a consequence of pathology. In contrast, some reactive oxygen species (ROS) may make a beneficial contribution to disease pathophysiology, for example, tissue injury may upregulate prostaglandins, interleukins and cytokines such as tumour necrosis factors (TNFs) (Halliwell and Gutteridge, 1999).

Increased free radical production has been implicated not only in disease processes, but also in exercise where the utilisation of oxygen can be manipulated. For example, systemic by-products of oxidative stress have been shown to increase as a result of physical exercise where oxygen flux has increased (Alessio *et al*, 2000) and decreased (Bailey *et al*, 2000). Again an association has been firmly established, however, researchers strive to find whether the radicals are formed as a result of tissue injury or whether the radicals cause the tissue damage (Jackson, 1996).

'Oxidative stress' is a popular term among exercise physiologists and clinical biochemists alike, and is widely used in the free radical literature, but rarely defined. Halliwell and Gutteridge (1999) define oxidative stress as *'a disturbance in the prooxidant-antioxidant balance in favour of the former, leading to potential damage'*.

Endogenous levels of oxidative stress, which may be both detrimental and beneficial to the human body, are controlled by the following mechanisms *in vivo*:

(A) Increased/decreased activity of the radical generating enzymes (*e.g.* xanthine oxidase) and/or their substrates (*e.g.* hypoxanthine);

(B) Activation/inactivation of phagocytes (*e.g.* neutrophils, macrophages and lymphocytes);

(C) Activation/inactivation of phospholipases, cyclooxygenases and lipoxygenases;

(D) Increase/decrease of antioxidants;

(E) Release/nonrelease of 'free' metal ions from sequestered sites and/or muscle;

(F) Release/nonrelease of haeme proteins (*e.g.* haemoglobin, myoglobin);

(G) Disruption/nondisruption of electron transport chain and increased electron leakage.

(Modified from Aruoma, 1994)

To fully understand the nature of damage inflicted on a biological system, a definition of a free radical must be established. Electrons in atoms circulate in pairs within a space known as *orbitals* (Halliwell *et al*, 1992). A free radical may simply be defined as any species (atoms, ions, or molecules) with one or more unpaired electrons in their outer atomic or molecular oribital, which are capable of existing independently (Halliwell and Gutteridge, 1999). Hence, the presence of an unpaired electron causes the species to be highly reactive. Furthermore, the occupancy of an unpaired electron causes the species to exhibit paramagnetism (*the property of reacting to an applied magnetic field*), which is the basis for detection by electron spin (paramagnetic) resonance spectroscopy (Ashton, 1998). Any reactive molecule with an unpaired electron is conventionally represented by the application of a superscript dot ($^{\cdot}$) (Halliwell *et al*, 1992).

Free radical interaction with biomembranes/molecules may be divided into three stages; initiation, propagation and termination. In a biological model, lipid peroxidation is often used to describe these sequential chain of events, where initiation involves the production of a free radical species with sufficient reactivity to abstract a hydrogen atom from a polyunsaturated fatty acid side chain (*such as those of arachidonic and linolenic acid*) (Halliwell and Gutteridge, 1999). Propagation is the production of subsequent free radicals as a result of molecular rearrangement, whilst termination is the result of two radicals

reacting with each other or scavenged by low molecular weight endogenous antioxidants (*e.g.* vitamin C) to result in a more stable molecule (Halliwell and Gutteridge, 1999).

Reactive Oxygen Species (ROS) is a collective term for oxygen-derived molecular species. Thus, this term includes free radical species, in addition to species that have paired electrons but are capable of becoming involved in harmful reactions that cause damage to other biomolecules (*e.g.* hydrogen peroxide and hypochlorous acid) (Halliwell and Gutteridge, 1999). The terms' reactive oxygen species, free radicals, radicals and oxidants are synonymous, and will be used interchangeably throughout this thesis.

Chapter 1 presents a review of the literature pertaining to free radical biochemistry and mechanisms of production *in vivo*. An overview of exercise-induced oxidative stress in apparently healthy and diseased populations will be given in order to draw a basis for the rationale for studies completed in this thesis. The latter sections will be mostly concerned with an overview of the antioxidant defence systems, in addition to an explanation of the pathogenesis of type 1 diabetes mellitus.

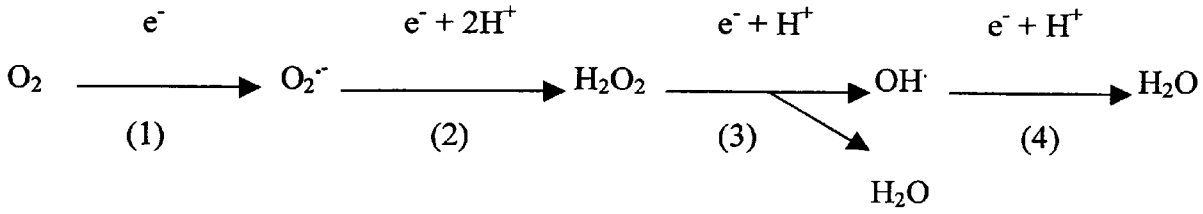
2.1 – Biochemistry of reactive oxygen species production in vivo

Free radicals are believed to be formed *in vivo* at rest and exercise as by-products of normal energy metabolism (Jackson, 1995). It has been estimated that for every twenty-five oxygen (O_2) molecules reduced by normal respiration, one free radical is formed (McCord, 1979). Given the fact that the rate of O_2 uptake by the body during exercise may increase up to approximately 35 fold (Aw *et al*, 1986) and that O_2 flux through active whole muscle tissue may reach 200 fold above resting values (Keul *et al*, 1972), it is possible that a rise in metabolism may increase free radical production, which may inevitably damage surrounding tissue and organs. This paradoxical '*oxygen*' relationship between an apparently healthy act (*exercise*) and the occurrence of harmful biological reactions (Kanter, 1998), prompted Jenkins (1993) to state that:

"Elemental and gaseous oxygen presents a conundrum in that it is simultaneously essential for and potentially destructive to human life".

During the process of complete tetravalent reduction of molecular oxygen to water, free radical intermediates and other toxic products are univalently formed (*figure 2.0*). The first one-electron reduction of molecular oxygen generates the superoxide anion radical ($O_2^{\cdot-}$). Addition of a second electron and two protons to superoxide will form hydrogen peroxide (H_2O_2). A third electron and one proton formulates the hydroxyl radical while adding another electron and a proton to hydroxyl will produce water (Davies, 1995). Superoxide, hydrogen peroxide and hydroxyl, the first three intermediary molecules generated in the pathway will be examined in more detail in the ensuing sections of this chapter.

Figure 2.0 - Univalent reduction pathway of molecular oxygen



One electron reduction = superoxide anion ($O_2^{\cdot -}$)
Two electron reduction = hydrogen peroxide (H_2O_2)
Three electron reduction = hydroxyl radical ($OH^{\cdot}$)
Four electron reduction = inert water (H_2O)

Adapted from Fridovich (1978)

2.1.1 – Oxygen and its derivatives

Arguably the most important free radicals in biological systems are radical derivatives from oxygen (Cheeseman and Slater, 1993). Although oxygen is a necessity for the survival of aerobes, it has the potential to become toxic when supplied at concentrations higher than normally encountered (Frank, 1985). The toxicity of molecular O_2 is largely due to the production of highly reactive oxygen species. Molecular O_2 is in fact a di-radical, having two unpaired electrons, located in a different antibonding orbital with the same directional spin. The subsequent effect of this arrangement is that oxygen can only react with non-radicals by accepting a pair of electrons that spin in an anti-parallel manner (McCord 1979, Young 1994).

Free radicals in living organisms include, superoxide ($O_2^{\cdot -}$), hydroperoxyl ($HO_2^{\cdot}$), hydroxyl ($OH^{\cdot}$), peroxyl ($RO_2^{\cdot}$), alkoxyl ($RO^{\cdot}$) and nitric oxide ($NO^{\cdot}$) radicals (*table 2.0*). Hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCL), as stated previously, have no unpaired

electrons, and by definition are not free radicals, but nevertheless are powerful oxidants that are often involved in free radical reactions (Halliwell 1989, Karlsson 1997).

Table 2.0 – Types of reactive oxygen species present in biological systems

Free radical	*Chemical Formula*	*Half-life (seconds)*
Hydroxyl	$OH^{\cdot}$	$1 \times 10^{-9*}$
Superoxide	$O_2^{\cdot -}$	$1 \times 10^{-6\#}$
Singlet oxygen	$^1O_2$	$1 \times 10^{-6\#}$
Alkoxyl	$RO^{\cdot}$	$1 \times 10^{-6*}$
Molecular oxygen	O_2	$> 10^{2\#}$
Hydrogen peroxide	H_2O_2	$10^{\#}$
Peroxyl	$ROO^{\cdot}$	7^{*}
Nitric oxide	$NO^{\cdot}$	8^{+}
Semiquinone	$Q^{\cdot -}$	Days*

*Source: * Pryor (1986); # Rimbach et al 1999; + Inoue et al 1999.*

2.1.2 – Superoxide anion

Superoxide ($O_2^{\cdot -}$) is a commonly known oxygen-centred free radical species. The reduction of a single electron to an O_2 molecule univalently produces the superoxide anion (*figure 2.0*) (Pryor, 1986). $O_2^{\cdot -}$ is relatively unreactive with non-radical species in comparison to other radical types (*e.g.* hydroxyl), however, if $O_2^{\cdot -}$ is generated near the site of any biochemical molecule it can be extremely destructive. The reactivity of $O_2^{\cdot -}$ differs in aqueous solutions and in organic solvents, but in general aqueous phase reactions are more likely to occur *in vivo* (Halliwell and Gutteridge, 1999). In this environment, $O_2^{\cdot -}$ can act as a base, accepting a proton to form the hydroperoxyl radical ($HO_2^{\cdot}$). The p*K*a *(ratio of concentrations at equilibrium)* for this reaction is 4.8, indicating that approximately only 1% of $O_2^{\cdot -}$ is in the $HO_2^{\cdot}$ form at physiological pH (Pryor, 1986).

Several mechanisms *in vivo* produce $O_2^{\cdot-}$, including, mitochondrial leakage, ischemia/reperfusion, auto-oxidation reactions, respiratory burst involving phagocytic cells and continuous production of $O_2^{\cdot-}$ by vascular endothelium to neutralise nitric oxide (Young and Woodside, 2001) *(section 2.2 for a more indepth account)*. $O_2^{\cdot-}$ under normal metabolic responses is dismutated by the enzyme superoxide dismutase (SOD), which increases the rate of intracellular dismutation by a factor of $10^{-9} \cdot M^{-1}s^{-1}$ to form hydrogen peroxide (H_2O_2) (Chance *et al* 1979, Hess and Manson 1984), as shown in the following equation;

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

This would suggest that $O_2^{\cdot-}$ is deliberately produced *in vivo*, as the presence of SOD has no other purpose other than to dismute $O_2^{\cdot-}$ to H_2O_2.

2.1.3 - Hydrogen Peroxide

Any biological system generating $O_2^{\cdot-}$ will produce H_2O_2 as a direct result of the dismutation reaction shown above. H_2O_2 is a relatively long-lived species (*table 2.0*) (Matsu and Kaneko, 2000). Thus, the longer half life allows H_2O_2 to pass freely through biological membranes (Young and Woodside, 2001), which $O_2^{\cdot-}$ generally cannot do without the aid of a negatively charged chloride (Cl^-) channel (Matsu and Kaneko, 2000). H_2O_2, can therefore act as a passage to transmit free radical induced damage across cellular compartments (Young and Woodside, 2001).

H_2O_2 is produced by a variety of intracellular reactions, although SOD is the main method. Several enzymatic reactions, including those catalysed by D-amino-acid oxidase in the peroxisomes and monoamine oxidase in the mitochondrial outer membrane may directly produce H_2O_2 (Sen *et al* 2000, Young and Woodside 2001). H_2O_2 in the presence of transition metals ions (*e.g.* copper and iron; fenton chemistry) can lead to the production of $OH^{\cdot}$, which is extremely reactive (Slater 1984, Blake *et al* 1987, Basaga 1990, Bast *et al*

1991), and probably the most reactive free radical known to chemistry (Tien *et al* 1982, Duthie *et al* 1989, Basaga 1990, Halliwell 1989).

2.1.4 - Hydroxyl Radical

The highly oxidising hydroxyl radical is probably the final intermediary product to be formed before tissue damage occurs (Lloyd *et al*, 1997). All of the reactive species summarised earlier exert most of their pathological effects by giving rise to hydroxyl radical formation (Young and Woodside, 2001). The reason for this is that the hydroxyl radical has the ability, once formed, to react immediately and abstract a hydrogen atom from many biological molecules, including sugars, DNA, lipids and thiols at an extremely high rate (Halliwell 1991, Young and Woodside 2001). The degradation of these compounds may produce damage products and a range of secondary organic radicals of variable reactivity (Halliwell and Gutteridge, 1999) that may possibly be measured directly by ESR spectroscopy (*e.g.* peroxyl, alkoxyl or alkyl radicals). However, due to the extremely high reactivity and short half-life of the hydroxyl radical, it may be unable to react with any molecule beyond 5 molecular diameters from the site of formation (Pryor 1986, Matsu and Kaneko 2000). Thus, the concept of site specificity is important, since if this radical is formed near DNA, it may damage any of the DNA bases, which may progress to the development of pathology. If this occurs, then the conclusion may be drawn, that the various antioxidant defences are uniformly ineffective in preventing hydroxyl radical induced damage (Young, 1994).

Hydroxyl radicals can be formed in various ways *in vivo* with homolytic and heterolytic bond fission being well-known sources (Halliwell and Gutteridge, 1999). However, by far the most widely known mechanisms of hydroxyl radical formation in biological systems is the transition metal catalysed decomposition of $O_2^{\cdot-}$ and H_2O_2 (Young, 1994), and these reactions are discussed in more detail in section 2.2.12.

2.1.5 – Reactive Nitrogen Species

Reactive Nitrogen Species (RNS) is a term used to explain the process of an unpaired electron residing on a nitrogen molecule (Halliwell and Gutteridge, 1999). Common RNS include nitrogen dioxide ($NO_2^{\cdot}$), nitrous acid (HNO_2), dinitrogen trioxide (N_2O_3), nitroxyl anion (NO^-), nitric oxide ($NO^{\cdot}$) and peroxynitrous acid (ONOOH). Of these, perhaps the most discussed RNS is $NO^{\cdot}$.

Nitric oxide ($NO^{\cdot}$) [*nitrogen monoxide*] is by definition a free radical due to the presence of a single unpaired electron (Sen *et al*, 2000). $NO^{\cdot}$ is produced in some mammalian cells, and can control blood flow, thrombosis and neural activity (Beckman and Koppenol, 1996). It has the ability to rapidly diffuse between cells and bind to $O_2^{\cdot-}$ to produce the peroxynitrite anion ($ONOO^-$), which although not a free radical, has the potential to attack and damage cellular membranes (Sen *et al*, 2000). One-electron reduction of $NO^{\cdot}$ would yield the nitroxyl anion, which is a relatively unreactive and short-lived species (Halliwell and Gutteridge, 1999).

However, perhaps the most important RNS (*also termed a ROS*) in biological systems is $ONOO^-$. This RNS is reported to have $OH^{\cdot}$ like characteristics, in that it can damage most molecules in its surrounding location (Beckman and Koppenol, 1996). Due to the lack of information regarding RNS in exercise and disease, and given the nature of the thesis presented, the author will predominately refer to the ROS from here on, unless otherwise stated. Excellent review articles on this topic by Beckman and Koppenol (1996) and Darley-Usmar *et al* (1995) are recommended.

2.2 – Mechanisms of reactive oxygen species production in vivo

Free radicals can be produced in cells and tissues by a variety of normal processes and reactions. The most widely documented sources of ROS formation *in vivo* will be discussed.

2.2.1 – Mitochondrial electron transport chain

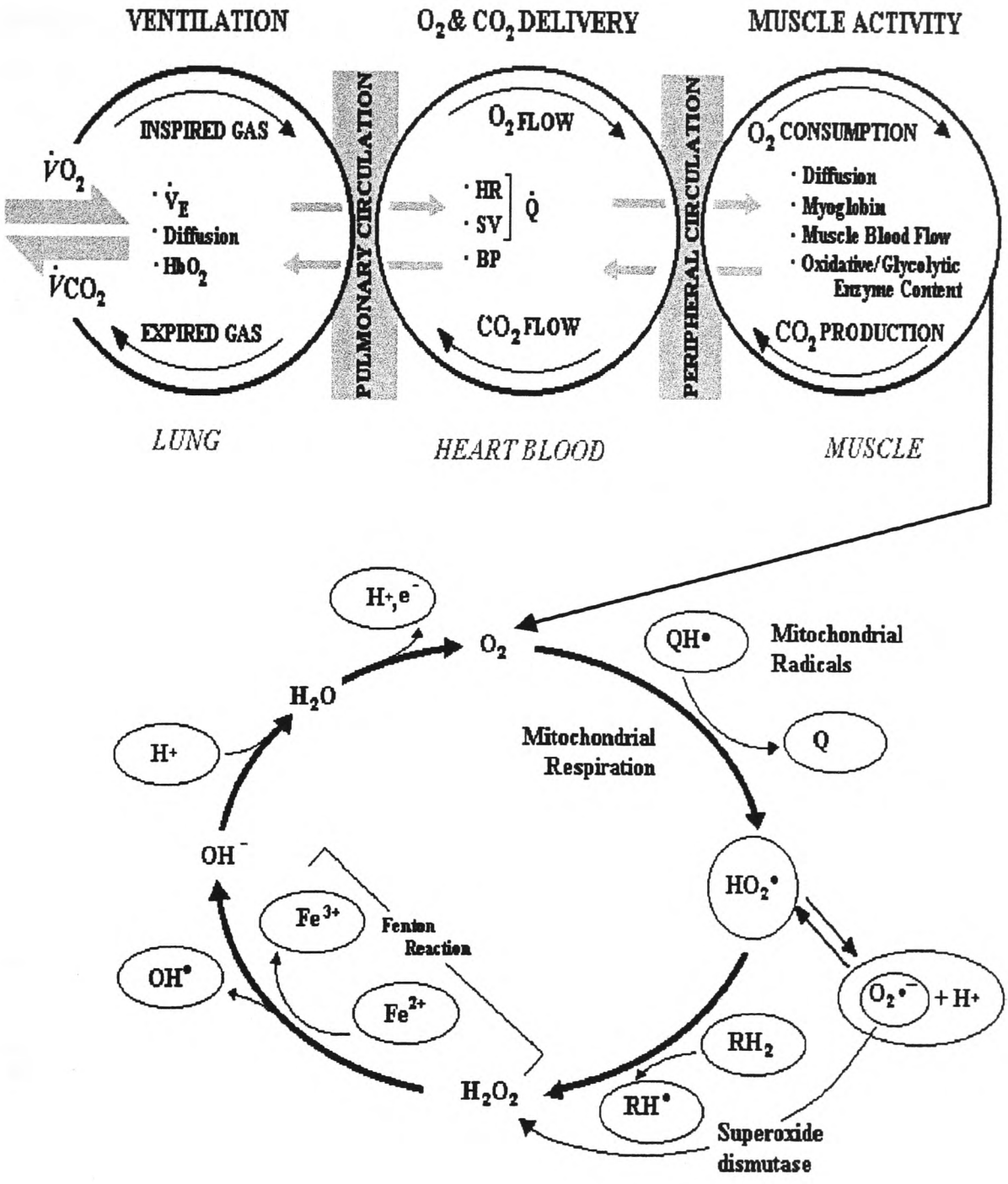
The mitochondia are the major site of oxidation and reduction of molecular O_2 to water (H_2O). The mitochondrial electron transport chain is the primary cellular source for adenosine 5'-triphosphate (ATP) generated via oxidative phosphorylation (Salway, 1998), and possibly the main source of cellular ROS formation (Sjödin *et al,* 1990).

In a normally functioning mammalian respiratory chain, electrons are passed from substrates in the citric acid cycle either directly from succinate or by the electron carrier NAD. The electrons pass through a sequence of protein or nonprotein transporters to the catalytic site of cytochrome oxidase, where 4 electrons together with 4 hydrogen ions tetravalently reduce molecular oxygen to H_2O (Sjödin *et al,* 1990). A proportion of the O_2 reduced by normal respiration is converted to $O_2^{\cdot-}$ at intermediate steps of the mitochondrial respiratory chain. This production may occur at the level of the NADH dehydrogenase (Turrens and Boveris, 1980) and/or at the level of ubiquinone-cytochrome bc_1 segment of complex III (Raha *et al*, 2000). There seems to be a disruption in the transfer of electrons from ubisemiquinone to the non-protein quinone, where electrons become dislodged and are 'freed' into the mitochondrial matrix where they bind with O_2 to form $O_2^{\cdot-}$ (*univalent reduction*) (Sjödin *et al,* 1990).

Estimates of resting percent leakage vary: from 1-4% (Richter, 1988), 2-5% (Boveris and Chance, 1973) to 15% by Sawyer (1988). This low rate of leakage is thought to be due to a low intra-mitochondrial concentration of oxygen (pO_2 = 1mmHg) (Halliwell and Gutterridge, 1999). Additionally, it has been estimated that for every twenty-five O_2 molecules reduced by the protein cytochrome oxidase, one free radical is formed (McCord, 1979). Therefore, if O_2 flux in active mammalian skeletal muscle can increase 200 fold (Keul *et al*, 1972), an increase in electron pressure/flux through rapidly respiring mitochondria could possibly lead to an enhancement of electron leakage and the formation of free radicals such as the quinone radical, superoxide anion and the highly reactive hydroxyl radical (*figure 2.1*) (Kanter 1998, Nohl and Hegner 1978).

The production of $O_2^{\cdot -}$ in the mitochondrial membrane is thought to be the precursor for the H_2O_2 observed in the mitochondria (Boveris and Cadenas, 1975). The rate of H_2O_2 formation in mitochondria is believed to be directly related to the energy coupling mechanism (Loschen *et al*, 1974). Therefore, greater biochemical demand from physical exercise, may enhance electron flux with subsequent electron leakage, thus, generating an increase in ROS and a simultaneous exercise-induced oxidative stress.

Figure 2.1 – Oxygen transport and utilisation pathway

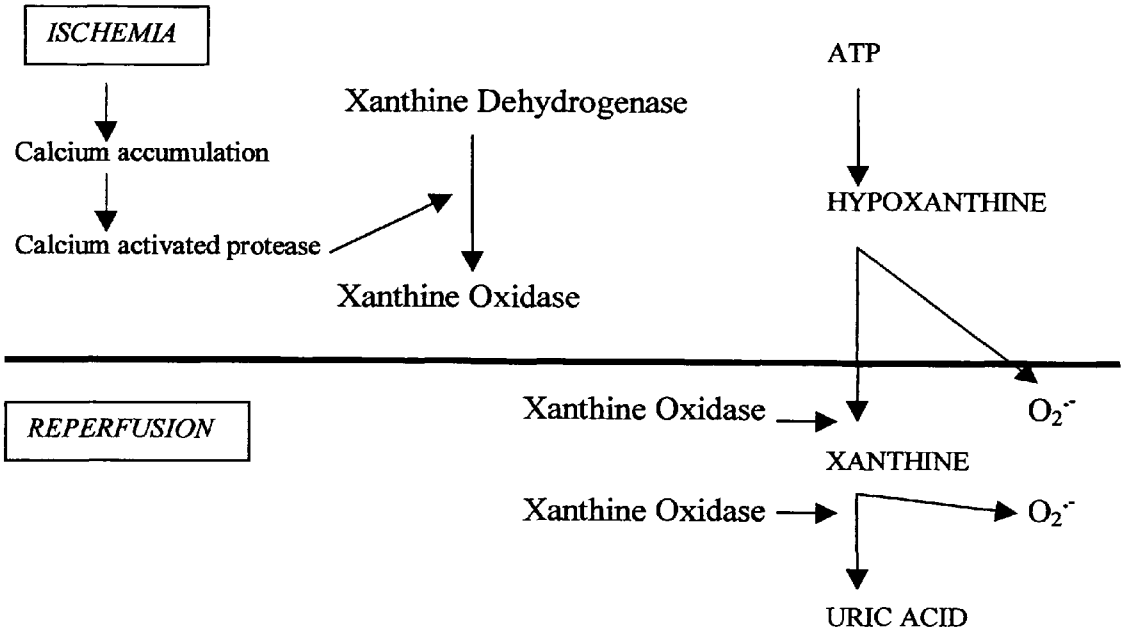


Diagrams modified from Wasserman et al (1994) (upper) and Packer (1997) (lower).

2.2.2 - Xanthine oxidase

Xanthine, an intermediary product of adenine nucleotide metabolism exists primarily in two enzymatic forms *in vivo*: xanthine dehydrogenase (XDH) and xanthine oxidase (XO) (Hellsten, 1994). In normal tissue, it is estimated between 70–90% of the total enzyme activity exists in the dehydrogenase form, located primarily in the vessel walls of many tissues including cardiac and skeletal muscle (Hellsten, 1996). Immunohistochemical studies using monoclonal antibodies to xanthine oxidase, have observed the presence of low concentrations of XO in the capillary endothelium of tissue such as liver, heart, lung and skelatal muscle (Sjödin *et al,* 1990). Under normal resting/metabolic conditions, XDH catalyses the oxidation of hypoxanthine to xanthine, and xanthine to uric acid, using nicotine amide dinucleotide (NAD^+) as the sole electron acceptor. However, when the body is subjected to heavy stress and ischaemia, it has been postulated that the configuration of the XDH enzyme changes to the irreversible oxidase form (XO), which upon blood reperfusion, uses molecular oxygen as the final electron acceptor, univalently forming superoxide radicals (Sjödin *et al* 1990, Hellsten 1996). The conversion mechanism of XDH to XO is largely unknown, however, a number of theories have been put forward, including the oxidation of critical sulphydryl (-SH) groups or by proteolysis (Halliwell and Gutteridge 1999, Hellsten 1996). Oxidation of the –SH groups may occur in response to lowered cellular thiol status, including glutathione, which occurs in ischaemia (Halliwell and Gutteridge, 1989). Roy and McCord (1983) suggest that the proteolytic cleavage of XDH during ischaemia is due to a lack of ATP regeneration, causing a malfunction to the ATP-dependent calcium pumps. The subsequent rise in intracellular calcium may activate calcium proteases (calmodulin) causing enzymatic cleavage (*figure 2.2*) (Sjödin *et al,* 1990).

Figure 2.2 – Schematic representation of free radical generation via xanthine oxidase during ischemia/reperfusion.



Adapted from Hellsten (1996)

Witt *et al* (1992) propose a theoretical mechanism linking intense exercise (*at or above* $\dot{V}O_{2\,max}$) with XO activated free radical production in skeletal muscle tissue. These authors speculate that during exercise the decrease in blood flow to organs and tissues (kidney and muscle) could result in hypoxia and upon cessation of exercise the return of re-oxygenated blood to these regions may lead to a '*respiratory burst*'. It is unlikely that myocytes become hypoxic, since cellular anoxia may be detrimental to the functioning of the human body.

Although exercise induced reperfusion injury is a popular concept (Armstrong 1986, Jakeman and Maxwell 1993), it appears unlikely that skeletal muscle could be damaged by such mechanisms. Subjects can rarely exercise for more than 2-3 minutes at $\dot{V}O_{2\,max}$, thus if muscle ischaemia occurs it is likely to be extremely transitory (Child, 1997). Mair *et al* (1995) found no evidence of free radical mediated injury following cardiac surgery and

concluded human striated muscle is extremely resistant to reperfusion injury. During such a procedure the severity and duration of occlusion is likely to be greater than experienced by skeletal muscle during exercise, thus, the possibility of exercise induced reperfusion injury appears remote. Additionally, Hellsten (1996) has shown raised levels of hypoxanthine in the blood of post-exercised individuals and concluded that hypoxanthine does not accumulate in the muscle during or after intense exercise, suggesting that hypoxanthine is rapidly released from the muscle after formation. This finding questions the possibility of intracellular free radical formation via an ischaemia/reperfusion mechanism.

2.2.3 – Auto-oxidation reactions

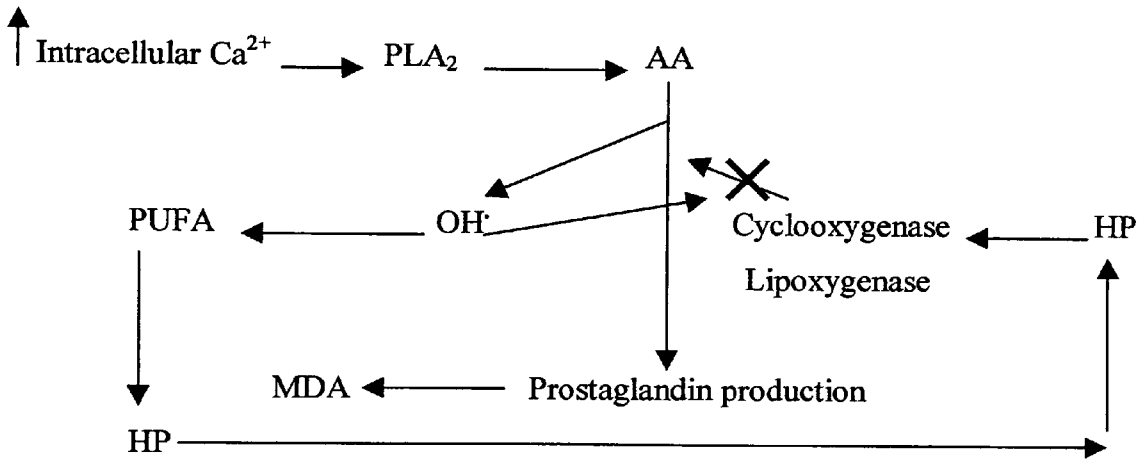
$O_2^{\cdot-}$ can also be generated by so called "*accidents of chemistry*" (Halliwell, 1996). Several molecules, including ascorbic acid, catecholamines, flavine nucleotides, tetrahydrofolates, glucose and cysteine can slowly oxidise in the presence of O_2 to form $O_2^{\cdot-}$, and this reaction is said to be greatly accelerated by the presence of iron or copper ions (Miller *et al* 1990, Young and Woodside 2001). The $O_2^{\cdot-}$ is subsequently capable of oxidising more of the originating compound, thus setting up a complex free radical chain reaction (Halliwell 1996, Young and Woodside 2001, Halliwell and Gutteridge 1999, Wolff 1993). Recent investigators have used ESR spectroscopy to measure haemoglobin autoxidation *in vivo* (Svistunenko *et al*, 1997).

2.2.4 - Prostaglandin production

The products of arachidonic acid metabolism include prostaglandins and thromboxanes and can provide a source of ROS *in vivo* (Halliwell and Gutteridge, 1989). Free radicals formed during prostaglandin synthesis appear to play a regulatory role. The enzymes cyclooxygenase and lipoxygenase, which oxidises arachidonic acid, requires hydroperoxides for its activation (Hemler and Lands, 1980). A radical, thought to be $OH^{\cdot}$ is formed which inhibits cyclooxygenase, hence providing a negative feedback control system for prostaglandin synthesis. This free radical production leads to lipid peroxidation,

which can provide the hydroperoxides required for the reaction to occur (Blake *et al*, 1987). Malondialdehyde (MDA), a product of lipid peroxidation, is also generated by this prostaglandin pathway (Halliwell and Gutteridge, 1999) (*figure 2.3*).

Figure 2.3 – Prostaglandin production pathway



Ca^{2+}, Calcium; PLA_2, Phospholipase A_2; AA, Arachidonic acid; OH·, Hydroxyl radical; PUFA, Polyunsaturated fatty acid; HP, Hydroperoxide; MDA, Malondialdehyde.

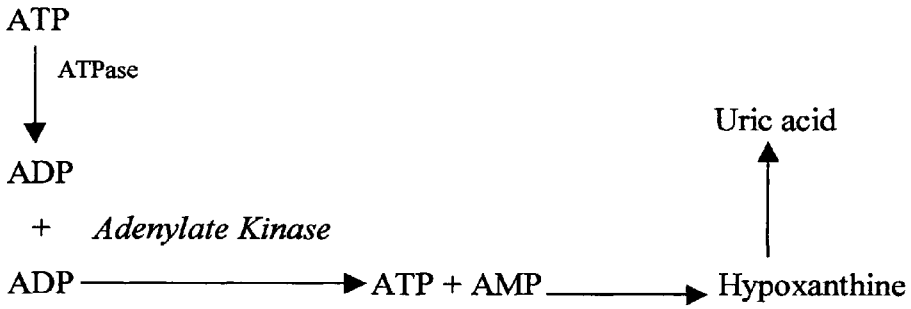
With relation to physical exercise, muscle injury may occur from strenuous or sporadic activity, increasing intracellular free calcium, which activates phospholipase A_2. This results in the accumulation of arachidonic acid which can produce prostaglandins and increase free radical formation (Bendich, 1991). Prostaglandins, serum lipid peroxides and malondialdehyde have all been shown to increase following acute exercise on a cycle ergometer (Viinikka *et al*, 1984).

2.2.5 – Adenylate kinase reaction

During exhaustive physical exercise, where the rate of ATP degradation may exceed that of regeneration, ADP and hydrogen ions may accumulate, activating the enzyme adenylate kinase (*or sometimes referred to as myokinase in muscle*) (Brooks *et al*, 1996). This

enzyme catalyses the conversion of two molecules of ADP to one molecule of ATP and AMP respectively (*figure 2.4*).

Figure 2.4 – Adenylate kinase reaction



ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate.

An intracellular accumulation of AMP can decompose into hypoxanthine, which is released into the vascular system forming uric acid as the end product of purine nucleotide metabolism. However, the production of xanthine oxidase during this process can generate $O_2^{\cdot-}$ radicals (Hellsten, 1996), as previously discussed.

2.2.6 – Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase

NADPH oxidase is found primarily in phagocytes and vascular endothelium cells, and is proposed to be an important source of free radical generation in muscle plasma membranes (Halliwell and Gutteridge 1999, Jackson 1994). The NADPH oxidase system is also considered an important $O_2^{\cdot-}$ source for cell-cell communication (Halliwell and Gutteridge, 1999). However, the physiological significance of this extracellular surface free radical production is largely unknown, and it is unclear whether this enzymatic system is influenced by exercise. Thus, due to the lack of research in this domain, this system as a potential source during exercise remains purely speculative (Jackson, 1994).

2.2.7 – Excessive calcium accumulation

Increased intracellular calcium (Ca^{2+}) concentration has been implicated in skeletal muscle damage and is thought to generate ROS, which may mediate pathological processes in cells (Jackson and Edwards, 1986). The mechanisms known to induce cellular damage to skeletal tissue has been the subject of much debate, and it is thought that a disruption in Ca^{2+} homeostasis is the primary reason for exercise-induced muscle damage (Jackson, 1994).

Hypoxic cells are known to deplete ATP which may cause the ATP-dependent Ca^{2+} pumps to malfunction (Sjödin *et al,* 1990). This occurrence may raise intracellular free Ca^{2+} content, which may activate Ca^{2+} proteases causing enzymatic cleavage to xanthine oxidase, which has a high affinity for O_2 as opposed to NAD^+, and produces superoxide radicals as a consequence (Sjödin *et al,* 1990) (*also see section 2.2.2*). This production in superoxide may lead to tissue damage and exercise-induced oxidative stress (Sjödin *et al,* 1990, Jackson 1994). In support, Jackson *et al* (1984) and Claremount *et al* (1984) discovered that the amount of cellular damage (*as measured by extracellular lactate dehydrogenase*) observed in isolated soleus muscle in response to hypoxic exposure was directly proportional to extracellular calcium concentration. Similar observations have been highlighted in cardiac tissue, where the degree of cellular damage induced by periods of ischemia-reperfusion also appears to be dependent on extracellular Ca^{2+} levels (Supinski *et al* 1999, Nayler 1981).

A rise in intracellular Ca^{2+} can activate endogenous enzymes, such as phospholipase A_2 causing cell membrane disruption. Within this process, Ca^{2+} causes the release of arachidonic acid from membrane phospholipids via the activation of phospholipase A_2, thus providing the substrate for increased prostaglandin production which is a recognised pathway for ROS production in skeletal muscle (Ashton 1998, Jackson 1998, Jackson and Edwards 1986) (*also see section 2.2.4*). The disruption to cell membranes may cause the efflux of many intracellular enzymes (*e.g.* creatine kinase, lactate dehydrogenase etc) (Jackson, 1998) supporting the notion of ROS production and subsequent cell injury in the

presence of increased Ca^{2+} ions. Studies of nimodipine, an L-type Ca^{2+} channel blocker have helped further examine the relationship between intracellular Ca^{2+} accumulation and free radical generation. For example, a recent study completed by Supinski and co-workers (1999), demonstrated that nimodipine added to contracting diaphragms suppressed ROS formation, suggesting that Ca^{2+} entry into the cellular compartment containing phospholipase A_2 may be an important factor in regulating free radical formation during muscle contraction. Furthermore, other Ca^{2+} antagonising agents have been shown to reduce creatine kinase release from normal skeletal muscle *in vitro,* (Anand and Emery, 1982), whilst other investigators have shown that interventions designed to reduce Ca^{2+} in skeletal muscle prevents the pathological damage to muscle in animals with congenital forms of muscular dystrophy (Bhattacharya *et al*, 1982). In contrast, treatment of skeletal muscle preparations with a Ca^{2+} ionophore (A23187) has demonstrated the potential of increased Ca^{2+} to induce damage (Johnson *et al,* 1988). This latter study incorporated ESR spectroscopy, and showed a 61% rise in peak height, accompanied by a large efflux of intracellular creatine kinase to the external medium. The radical species found is thought to be semiquinone, derived from calcium overload within the mitochondria.

It is evident that cellular deformation and disruption of Ca^{2+} homeostasis are closely associated with oxidative stress and are important components of the damage caused by free radicals. As with other mechanisms there still remains the challenge in determining cause and effect with reference to the relationship between intracellular Ca^{2+} accumulation and exercise-induced oxidative stress.

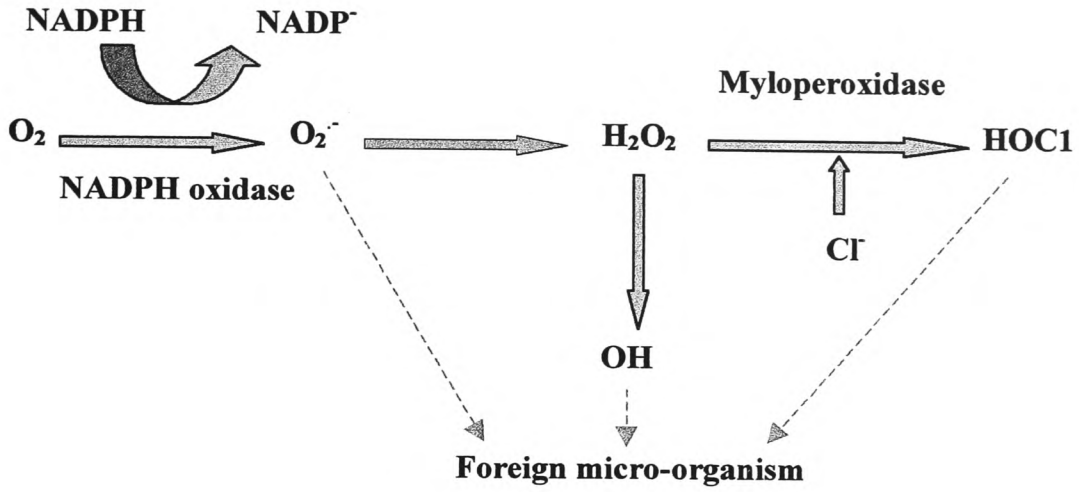
2.2.8 – Respiratory burst

When phagocytic cells, such as neutrophils, monocytes, eosinophils and macrophages encounter foreign particles (*e.g.* bacteria), they become activated and produce $O_2^{\cdot-}$, H_2O_2 and $OH^{\cdot}$, as a result of a series of reactions termed the *'respiratory burst'* (Blake *et al* 1987, Halliwell 1997, Brown 1993).

There are two known mechanisms that detail the process of bacterial scavenging by ROS production. During phagocytosis, a membrane bound oxidase, thought to be NADPH oxidase is activated, leading to an increased uptake and reduction of molecular oxygen to $O_2^{\cdot-}$ (Pal Yu 1994, Gordon *et al* 2000) (*figure 2.5*). Much of this respiratory activity takes place within the hexose monophosphate shunt which provides NADPH as the electron donor for the reduction of molecular O_2 to $O_2^{\cdot-}$ (Gordon *et al* 2000, Blake *et al* 1987). However, although this system is present in all human species, not all micro-organisms are destroyed. Patients with *chronic granulomatous disease* (CGD), an inherited condition in which the membrane-bound NADPH oxidase system in phagocytes is dormant (*phagocytosis is normal but the respiratory burst is absent*), show multiple and increased incidence of infection by some bacterial strains (*especially Staphylococcus aureus*), whose killing by neutrophils requires oxygen (Halliwell 1991, Halliwell and Gutteridge 1999).

The other existing mechanism used exclusively by neutrophils, involves the enzyme myeloperoxidase. Within the phago-lysosome, $O_2^{\cdot-}$ is spontaneously dismutated by extracellular SOD to H_2O_2 which interacts with the halide Cl^- ion in the presence of myeloperoxidase to form hypochlorous acid (HOC1), a powerful antibacterial agent (Blake *et al,* 1987) (*figure 2.5*). In contrast, phagocytosis in the presence of the antioxidant enzymes, catalase or superoxide dismutase may also reduce bactericidal activity (Chance *et al,* 1979). In addition to H_2O_2 formation, $OH^{\cdot}$ radicals may also be produced during phagocytosis, arising from either the Haber-Weiss reaction or the iron-catalyzing Fenton reaction, in which H_2O_2 is oxidised to $OH^{\cdot}$ by Fe^{2+} (Pal Yu, 1994). In support, Cohen *et al* (1991) utilised ESR to determine hydroxyl radical involvement in inflamed human mononuclear phagocytes, and found that these radical species may play an important role in this cell type.

Figure 2.5 – Production of ROS by the NADPH oxidase and myeloperoxidase mechanism during the 'respiratory burst'.



Although this deliberate source of ROS production is an important immune defence mechanism, it can also be a potentially dangerous mechanism if inappropriately activated (Halliwell, 1997). For example, overproduction of ROS may cause unwanted damage to adjacent tissues and molecules (Martinez-Cayuela, 1995). This seems to be the case in patients with *chronic inflammatory diseases* such as rheumatoid arthritis. In this condition, there may be a translocation of phagocytes and subsequent overactivation of toxic ROS causing increased damage to the already inflamed arthritic joint (Halliwell, 1997).

Strenuous exercise is normally followed by an increase in circulating neutrophils and other phagocytic cells, and in part, may be termed the '*acute phase immune repsonse*' (Jackson 1998, Cannon and Blumberg 1994). Since exercise is also known to increase susceptibility to infection, a disturbance of the immune system may provide an important source of ROS production during exercise of particularly long duration (Ashton 1998, Nieman 1994). Downhill running which involves predominately damaging eccentric actions has been found to cause greater neutrophilia than uphill exercise at the same intensity (Smith *et al*, 1989), In contrast, Miyazaki *et al* (2001) has shown that 12 weeks of high-intensity

endurance training can decrease neutrophil $O_2^{\cdot-}$ production in response to exhaustive exercise.

It is also relevant to point out that because tissue may become damaged during exercise, there may be an upsurge in phagocytic white cells, causing an associated increase in ROS production. However, Jackson (1998) calls for caution, and claims that this increase in free radical concentration does not equate to an '*exercise-induced*' increase, although there may be an analogous rise in by-product markers.

2.2.9 – Peroxisomes

Peroxisomes are membrane-bounded vesicles that contain a number of oxidative enzymes including, glycolate oxidase and urate oxidase, which produce H_2O_2 but not $O_2^{\cdot-}$. (Boveris *et al*, 1972). Peroxisomes contain large quantities of the enzyme catalase, which metabolises most of the H_2O_2. Some CuZnSOD may also be present in peroxisomes (Fridovich, 1995). There exists some controversy over the amount of H_2O_2 that leaks into the cytosol (Chance *et al*, 1979), which will reflect the extent of possible damage that ensues.

2.2.10 - Endoplasmic reticulum

Cytochromes P_{450} and b_5 present in the endoplasmic reticulum and nuclear membranes respectively, are involved in cellular demethylation, hydroxylation, and desaturation reactions of endogenous proteins and lipids (Freeman and Crapo, 1982). They demonstrate their optimum activity in the presence of NADPH and NADH respectively. However, under certain conditions, uncoupling may occur, in that O_2 is reduced to $O_2^{\cdot-}$ and H_2O_2, as opposed to being added to the substrate for complete coupling (Halliwell and Gutteridge, 1999). Cytochrome P_{450} is probably the main source of radical production in membranes of the endoplasmic reticulum (Chance *et al*, 1979).

Theoretically, under conditions of increased exercise intensity, the endoplasmic reticulum may provide an elevated source of free radical production, due to increased O_2 flux and subsequent electron *'leakage'* in the cytochrome P_{450} pathway.

2.2.11 - Nitric oxide synthase

Increased activity of skeletal muscle is known to be associated with a marked increase of $NO^{\cdot}$ production and release by the tissue (Balon and Nadler, 1994). As previously stated $NO^{\cdot}$ can bind to $O_2^{\cdot-}$ to form $ONOO^{-}$. In contrast, little is known with regards to $ONOO^{-}$ and its relationship with exercise, but it is a product that will inevitably be formed during physical exercise.

2.2.12 – Transition metals and other mechanisms of free radical formation

Other mechanisms exist *in vivo* that produce the reactive hydroxyl radical, either through the Haber-Weiss reaction or an iron-catalysed Fenton reaction (Halliwell and Gutteridge, 1999).

Fritz Haber first proposed in 1934 (Haber and Weiss, 1934), that hydroxyl radicals were produced when superoxide came in contact with hydrogen peroxide, as outlined in the following reaction:

$$O_2^{\cdot-} + H_2O_2 \longrightarrow O_2 + OH^{\cdot} + OH^{-}$$

This reaction, which created a great deal of attention at the time, was found to be too slow to be important in medicine and biology (Wardman, 1993). This underlines the importance of considering not only the products or course of a reaction, but also its rate.

Similarly, Henry Fenton in 1894 (Quoted by Young and Woodside, 2001) discovered that by adding hydrogen peroxide to the reducing agent, ferrous iron (Fe^{2+}), organic compounds could be oxidised (Halliwell and Gutteridge, 1999). Partly as a result of studies conducted

in the 1940s, the reaction is now known to involve the generation of the hydroxyl radical, and was subsequently named the Fenton reaction (Halliwell and Gutteridge, 1999). The reaction sequence is as follows:

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\cdot} + OH^{-}$$

Copper can also be involved in catalysing the Haber-Weiss reaction (Halliwell and Gutteridge, 1999). Therefore, the intracellular levels of these transition metal ions are critical in defining the extent of hydroxyl radical production from superoxide and hydrogen peroxide. In contrast, it is not the average concentration that may be important, but where within the cell the ions are located, since free iron and hydrogen peroxide will generate hydroxyl radicals that could damage DNA located in the same vicinity.

However, it is debatable whether Fenton chemistry occurs *in vivo* due to the presence of the storage proteins, ferritin and caeruloplasmin, which tightly bind iron and copper respectively, inhibiting free radical generation. This '*sequestration*' of metal ions has been reported to be an important antioxidant defence mechanism (Halliwell, 1996).

2.3 – Reactive oxygen species (ROS) production and exercise

Jenkins (2000), estimates that approximately 642 studies have been published since 1985 that have used the terms oxidative stress and exercise, and most, but not all claim that exercise can cause oxidative damage to important biomolecules, including lipids, proteins and nucleic acids. Research involving exercise-induced oxidative stress and related pathology is scarce, and the few studies that have been performed have failed to use more than one marker of oxidation, thus the unequivocal claim that exercise is associated with increasing oxidative damage and disease must be questioned.

Many methodological techniques have been adopted to quantify oxidative stress/damage as a result of physical exercise and pathology. These include the indirect by-products of lipid, protein and DNA oxidation. Electron Spin Resonance (ESR) spectroscopy is arguably the

most sensitive, specific, and direct method of measuring free radical species and is currently under-utilised in the clinical and physiological domain (Ashton *et al*, 1999).

The following section will review the pertinent studies relating to the direct and indirect measures of oxidative stress and exercise with relation to the healthy and diseased model.

2.3.1 – Indirect evidence of oxidative stress and exercise in healthy and pathological models

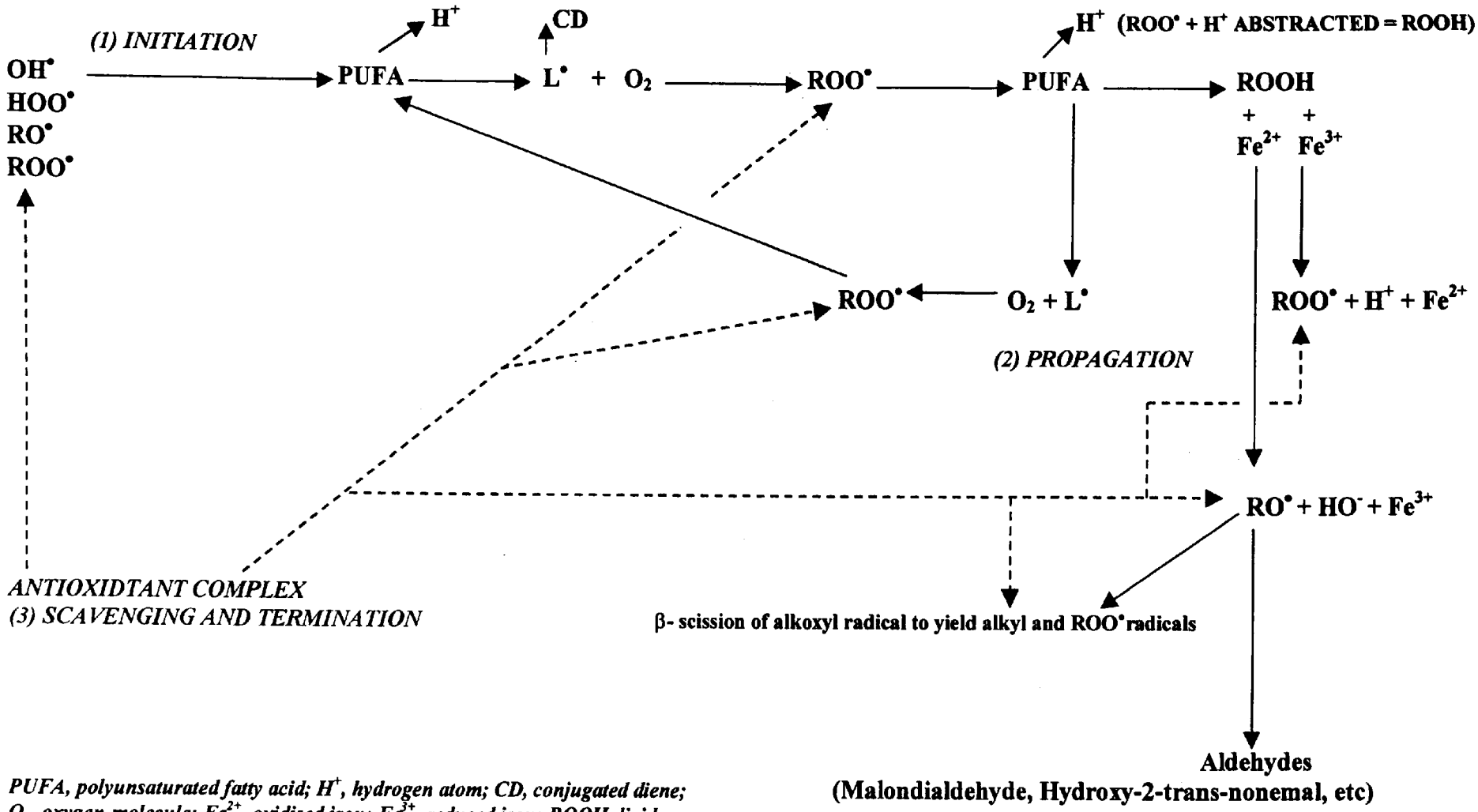
The by-products of lipid peroxidation are arguably the most studied indicators of exercise-induced oxidative stress (Kanter 1998, Jenkins 2000). This is perhaps due to the relative ease of assay techniques available to investigators (Jenkins, 2000). The by-products of lipid peroxidation are formed at various stages of the lipid oxidative process (*figure 2.6*), and include in order of production, conjugated dienes, lipid hydroperoxides, malondialdehyde and 4-hydroxynonenal. Measures of breath hydrocarbons have also been used to quantify lipid damage.

Although many markers of lipid peroxidation exist, there are still inconsistent reports in establishing the relationship between lipid peroxidation and exercise. Interpretation of existing data is difficult and this is due to the diversity of exercise protocols, fitness status, and the analytical methodologies employed (Jenkins, 2000). The latter is particularly relevant to the non-specific TBARS assay.

Following is an overview of various studies that have applied the by-products of lipid peroxidation to examine exercise-induced oxidative stress in healthy volunteers.

2.3.1.1 - Healthy model

Conjugated dienes are polyunsaturated molecules with two double bonds separated by a single bond (Alessio, 1993), and are rapidly formed once membrane peroxidation has been initiated (Pryor and Castle, 1984). Conjugated dienes in the presence of oxygen can form



PUFA, polyunsaturated fatty acid; H^+, hydrogen atom; CD, conjugated diene; O_2, oxygen molecule; Fe^{2+}, oxidised iron; Fe^{3+}, reduced iron; ROOH, lipid hydroperoxide; $OH^{\bullet 1}$, hydroxyl; $HOO^{\bullet 1}$, hydroperoxyl; $ROO^{\bullet 1}$, peroxyl; $RO^{\bullet 1}$, alkoxyl; $L^{\bullet 2}$, lipid radical; Alkyl radical[2]; 1 = oxygen centred radical, 2 = carbon centred radical. —— = lipid peroxidation pathway; - - - = antioxidant interaction

Figure 2.6- Lipid peroxidation chain of events and antioxidant interaction

lipid peroxyl radicals which can attack and cause one or more double bond molecules to shift to become diene conjugated (Jenkins 2000, Alessio 1993).

Duthie *et al* (1990) showed no change in conjugated dienes following a half marathon, however, Marzatico *et al* (1997), found conjugated dienes levels to be significantly increased in six ($n = 6$) marathon runners competing in a half-marathon event. Vasankari *et al* (1997) examined six top level skiers during a 30 km race at moderate altitude (1650 m) and found no change in serum conjugated dienes from pre- to post-exercise. Although conjugated diene levels were found to be significantly higher during exercise at altitude than at sea level.

Levels of conjugated dienes have not only been measured in blood following exercise but in other biological samples. For example, Merry *et al* (1991) observed no difference in conjugated dienes measured in synovial fluid between subjects participating in level walking or performing isometric contraction. It appears that the exercise literature reporting on conjugated dienes is inconclusive. This may be related to the non-specific assay used in measurement (Haramaki and Packer, 1994). Furthermore, it has been reported that only 30-55% of lipid peroxidation is actually detected by diene measurement, thus its importance as a marker of *in vivo* lipid damage during exercise must be addressed (Alessio, 1993).

Lipid hydroperoxides (LH) are considered to be the first major by-products of lipid oxidative metabolism (Ashton *et al*, 1999). They are formed by the abstraction of a hydrogen atom from a fatty acid side chain usually by the peroxyl radical (Halliwell and Gutteridge, 1999). Lipid hydroperoxides are relatively unstable in the presence of metal ions, and may decompose to a variety of reaction products including malondialdehyde (Haramaki and Packer, 1994).

Few studies have used lipid hydroperoxides as a marker of exercise-induced oxidative stress. Ashton *et al* (1998) reported an increase in serum LH ($P < 0.05$) in 12 non-trained healthy men after a one-off bout of exhaustive exercise on a cycle ergometer. However,

Viinikka *et al* (1984), showed no change in LH values from pre-to post-exercise on a cycle ergometer in 10 well-trained individuals. These findings would suggest that the appearance of LH during exercise is partially controlled by the training status, and more specifically by the enzymatic antioxidant status of the subjects used.

Viguie *et al* (1993) failed to detect an increase in LH after 90 mins of cycle ergometry at 65% $\dot{V}O_{2peak}$ in rats. In contrast, Baker *et al* (2000) has shown an increase in LH concentration following a 30 sec maximal exercise test on a cycle ergometer ($P < 0.05$).

Using a novel approach, Alessio *et al* (2000) compared LH in 12 subjects before and immediately after exhaustive aerobic (treadmill run) and isometric exercise (hand grip dynamometer). Oxygen consumption was found to be six times higher in the aerobic exercise than in the isometric exercise. LH was shown to increase significantly post isometric exercise but not after the aerobic exercise challenge. In a similar study, where systemic oxygen flux was also greatly reduced as measured by arterial oxygen saturation (SaO_2), Bailey *et al* (2000) showed a greater LH concentration in eighteen (n =18) active students performing a cycling test to volitional exhaustion in normobaric hypoxia (F_IO_2 = 16%) in comparison to exercise in normobaric normoxia (F_IO_2 = 20.9%). Furthermore, the hypoxic exercise-induced increase in LH was consistently associated with a decrease in SaO_2. These data would strongly support the hypothesis that a mass action effect of systemic $\dot{V}O_2$ is not the only mechanism responsible for an increase in exercise related oxidative stress. Additionally, it has been suggested that a decrease in mitochondrial PO_2 rather than an increase in mitochondrial O_2 flux may prove to be a more accurate mechanism of mitochondrial and/or systemic free radical generation (Bailey, 2001). Although there is clear indirect evidence of increased oxidative stress during exercise while inhaling a reduced concentration of oxygen, no group has yet been concerned with measuring free radical activity directly (*using ESR spectroscopy*) during exercise in a hypoxic environment.

Malondialdehyde (MDA) is frequently reported as a measure of exercise-related change to lipid cell membranes (Child *et al* 1998, Kanter *et al* 1988).

Research has recently been conducted using an endurance protocol to determine the effects of prolonged whole body oxygen exposure on lipid peroxidation production. Child *et al* (1998) examined MDA in seventeen (n = 17) trained runners before and after a simulated half marathon. All subjects ran on a treadmill at predetermined speeds corresponding to 50% $\dot{V}O_{2peak}$ for 5 mins and 70% $\dot{V}O_{2peak}$ for 10 min. Thereafter, the subjects were allowed to adjust their own treadmill speed and were verbally encouraged to complete the pre-set distance in the shortest possible time. Oxygen uptake increased as exercise progressed, peaking at the 20km mark. MDA, measured via HPLC significantly increased ($P < 0.05$) from rest (1.48 $\pm$ 0.39 $\mu mol.L^{-1}$) to immediately post-exercise (1.65 $\pm$ 0.32 $\mu mol.L^{-1}$). Markers of muscle damage were also measured (creatine kinase and β-glucuronidase) and were shown to increase following exercise.

In an earlier study, Kanter *et al* (1988) documented a near two-fold increase in serum MDA of nine (n = 9) male subjects following an eighty kilometer race. In agreement with Child *et al* (1998) a rise in serum creatine kinase post-exercise was shown, and found to correlate with MDA. Collectively these results indicate that whole body oxygen uptake can increase free radical production perhaps via mitochondrial electron '*leakage*', causing lipid peroxidation which in turn can compromise the integrity of the cell membrane.

Lovlin *et al* (1987) designed a study to determine whether the indices of free radical damage were related to exercise intensity. Six (n = 6) male students volunteered to cycle to volitional exhaustion on a cycle ergometer and give blood at selected intervals throughout the test. The protocol included a 5 min warm up period while resistance was increased (30W) every minute thereafter until exhaustion. Results indicated a 26% increase in MDA concentration from rest to immediately post-exercise ($P < 0.05$). Throughout exercise, MDA was shown to decline significantly at 40% $\dot{V}O_{2\,max}$ and was still found to be below resting concentrations at 70% $\dot{V}O_{2\,max}$. With a similar hypothesis, Sen *et al* (1994) measured MDA following sub-maximal exercise and reported a 50% and 100% increase at the corresponding aerobic and anaerobic threshold. Likewise, Kanter *et al* (1993) found serum MDA to rise following exercise at 90% $\dot{V}O_{2\,max}$.

It is reasonable to suggest based on the aforementioned findings that MDA production is regulated by the intensity of the exercise challenge. Thus increased mitochondrial oxygen uptake and subsequent electron leakage may form free radical species, which possibly attack lipid double bond structures and produce MDA.

Maughan *et al* (1989) measured MDA via the TBARS method before and following 45 mins of down hill running, and reported a significant rise in TBARS peaking at 6 hrs within a 72 hour post-exercise period. This would suggest that a continuous '*oxidant*' insult to cell membranes is occurring after the cessation of exercise, or a lag time in blood MDA appearance. Additionally, investigators have been criticised for the use of the TBARS assay in measuring MDA, with the underlying problem being that MDA is formed from pathways other than lipid peroxidation (Alessio, 1993). However, MDA may be measured more accurately and reliably with the use of more sophisticated techniques. For example MDA was measured in the studies outlined in this thesis by high performance liquid chromatography (HPLC).

Others have also found an increase in MDA/TBARS concentration following exercise in both the human and animal model (Ashton *et al* 1998 1999, Bailey *et al* 2000, Suzuki *et al* 1983, Alessio *et al* 1988, Davies *et al* 1982).

Investigators have not only examined TBARS post acute-exercise, recently a training study by Miyazaki *et al* (2001), have shown that 12 weeks of running at 80% maximal heart rate for 60 min · day^{-1} helped decrease both resting and exercise induced erythrocytes TBARS levels in untrained subjects. Furthermore, high-intensity endurance training was shown to up-regulate erythrocyte antioxidant enzyme activity, which would help explain the attenuation of exercise-induced lipid peroxidation. In constrast, Tharp *et al* (1995), used a similar design to the previous study (8 weeks of cycling at 85% maximal heart rate for 30 min · day^{-1}) and showed no change in urine MDA levels after a maximal exercise bout. However, resting urine MDA levels were found to increase during the course of eight weeks, suggesting that chronic aerobic exercise may produce a cumulative increase in lipid

peroxidation or the same production with a decrease in antioxidant status. Unfortunately the authors failed to report antioxidant status.

Krotkiewski and Brzezinska (1996) examined vastus lateralis muscle tissue immediately after 90 min of strenuous exercise in 27 untrained subjects. Additional blood samples were drawn before and repeatedly after exercise (immediately and 5 min post, 24 and 48 hrs post). O_2 uptake measured was shown to increase as exercise progressed. Plasma MDA levels increased immediately post-exercise and were shown to decline after 5 mins, remaining at this same level until the 48 h mark. Muscle tissue MDA values were much higher than the plasma concentration immediately post-exercise. The content of MDA in muscle tissue was found to correlate positively with the percentage of type I muscle fibres and negatively with both type II and IIB fibers. Similarly, a positive and negative correlation was observed for MDA concentration in type I and II muscle fiber cross-sectional area respectively. This data would suggest that the production of MDA parallels the exercise-induced increase of O_2 uptake in muscle tissue, and is highest in more oxidative consuming muscle fibers.

To conclude that oxidative stress has not occurred in the studies that have failed to detect a rise or have shown a decrease in MDA concentration following exercise is somewhat presumptuous according to Jenkins (2000). It has been shown that TBARS are quickly cleared from electrically stimulated muscle, and that rodents exercised to exhaustion showed a significant increase in urinary TBARS (Jenkins *et al,* 1993). Therefore, the failure to detect an increase in MDA that has actually been produced as a function of exercise, includes such potential factors as MDA production and clearance and therefore a failure to look at the right time or place (Jenkins, 2000).

The aforementioned data would suggest that malondialdehyde production is partially regulated by an O_2 dose-dependent mechanism, therefore, eliciting large increases in whole body O_2 uptake during exercise can cause significant damage to lipid cell membranes. This may be supported by data from Ashton *et al* (1998) showing a significant positive correlation between $\dot{V}O_{2\,max}$ and MDA post-exercise.

Hydrocarbon breath tests

Ethane and pentane are derived from *n*-3 and *n*-6 polyunsaturated fatty acids respectively, and can be detected in expired breath in increased amounts as a result of polyunsaturated fatty acid peroxidation (Halliwell and Gutteridge, 1999). Pentane was first used as a measure of lipid peroxidation in exercise by Dillard *et al* (1978), where a 1.8-fold increase was observed in six subjects following 20 min of cycling at 75% $\dot{V}O_{2\,max}$. This technique is often criticised because investigators fail to consider pentane levels in the atmosphere that may interfere with exercise samples. Dillard and colleagues used a spirometer to eliminate high background pentane levels (detection of 18-40 pmol/100ml room air). The authors suggest that the possible source of pentane is via the peroxidation of lipid microsome membranes, particularly that of the liver. This implies that pentane can travel through the systemic circulation and readily diffuse into the region of the lung where it is exhaled.

It has been shown that breath hydrocarbons are formed in parallel with exercise intensity. In a study by Leaf *et al* (1997) expired ethane and pentane levels were increased above resting levels at lactate threshold and continued to rise at $\dot{V}O_{2\,max}$. In agreement with these finding, Kanter *et al* (1993) found that expired pentane levels doubled after 30 min of exercise at 60% $\dot{V}O_{2\,max}$ (*rest*, 12.4 pmol·kg^{-1}·min^{-1} *vs. exercise*, 24.6 pmol·kg^{-1}·min^{-1}) and further increased after exercise at 90% $\dot{V}O_{2\,max}$ (33.4 pmol·kg^{-1}·min^{-1}). These results support previous comments in that *in vivo* lipid peroxidation may be controlled by the severity of the exercise challenge.

It is pertinent at this point to emphasise that the hydrocarbons are minor end-products of lipid peroxidation, and their formation is dependent upon the presence of iron or copper ions to decompose the peroxides (Halliwell and Gutteridge, 1999). Therefore, an increased rate of ethane and pentane gas production may reflect an increase in the availability of these transition metal ions rather than an increase in lipid peroxidation (Halliwell and Gutteridge, 1999).

For many years, investigators have widely documented the health benefits of physical exercise. An overview of the oxidative stress literature would suggest that exhaustive exercise does indeed cause damage to the cell lipid membrane and recent evidence has shown that exercise can modify DNA molecules (Poulsen *et al*, 1996). Therefore, one must consider whether exercise in its entirety is good for human health. This has prompted Jenkins (2000), to ask the pertinent question; can exercise induce an oxidant stress that might exert potentially adverse effects on human health? Undoubtedly, much more research applying a broader spectrum of analytical tools is needed to make conclusive statements relating exercise and oxidative stress to pathology. However, Jenkins (2000) proposes that aerobic exercise for prolonged portions of the human life span is a recent phenomenon that began in the early 1960s, and because the incubation period for disorders such as certain cancers is 30-40 years, the effects of continued oxygen exposure might yet appear.

2.3.1.2 - Pathological model

Comparatively few studies have been published examining the role of exercise-induced oxidative stress in the diseased model. Heunks *et al* (1999) recently measured lipid peroxidation (MDA) pre- and 60 min post-exercise in sixteen (n =16) chronic obstructive pulmonary disease (COPD) patients with (n = 8) and without (n = 8) prior treatment of the xanthine oxidase inhibitor, allopurinol. Reduced (GSH) and oxidised glutathione (GSSH) was also measured. All patients were instructed to exercise to exhaustion on a cycle ergometer (approximate workload = 75 W). In non-treated COPD patients, maximal exercise resulted in a significant increase in the GSSG:GSH ratio and in MDA (0.68 $\pm$ 0.08 nmol·ml at rest to 1.32 $\pm$ 0.13 nmol·ml post-exercise) concentration. In contrast, there was no change in MDA (0.72 $\pm$ 0.15 nmol·ml at rest to 0.64 $\pm$ 0.09 nmol·ml post-exercise) or in the GSSG:GSH ratio post-exercise in the patients treated with allopurinol. The authors conclude that strenuous exercise in COPD patients' results in lipid peroxidation, which can be inhibited by allopurinol treatment. This indicates that xanthine oxidase is an important source for free radical generation during exercise in COPD. Unfortunately, Heunks *et al* (1999) did not include a healthy control group in their study, which would have allowed a

comparison to be made with healthy exercising subjects. However, in a non-exercise study, Sahin *et al* (2001) showed that eighteen (n = 18) patients with COPD had significantly higher MDA levels at rest that those without the disease (2.68 $\pm$ 1.28 nmol·ml *vs.* 1.04 $\pm$ 0.36 nmol·ml).

Exercise-induced lipid peroxidation was measured in eight (n = 8) patients with myocardial ischemia, and in eight controls without myocardial ischemia (Leaf *et al*, 1998). All subjects were required to participate in an exhaustive exercise stress test and were subjected to thallium imaging in order to assess myocardial ischemia. Pre- and post-exercise MDA concentration was assayed via the HPLC method. Results showed a 46% increase in MDA levels in the patients with myocardial ischemia and a 16.8% decrease in MDA in the control group. Halliwell and Gutteridge (1999), suggest that two or more markers of lipid peroxidation are required in order to claim that an oxidative stress has occurred. This study used only one marker of lipid peroxidation and failed to correct the data for plasma volume changes, thus, the data must be viewed with caution. Likewise, Bardin *et al* (1987) used only one by-product of lipid peroxidation in order to test the effects of cycle ergometry in 43 ischemic heart disease patients. No control group was included in this study. The authors found an increase in MDA post-exercise and proposed that natural antioxidants be used to prevent myocardial metabolic disorders in coronary patients.

D'Inca *et al* (1999) found no change in plasma MDA levels in six (n = 6) male patients with Crohn's disease when compared with six (n = 6) healthy controls after a one-hour bout of exercise at 60% $\dot{V}O_{2\,max}$. However, the antioxidant trace element Zinc decreased after exercise in the Crohn's disease patients and remained unchanged in control subjects. In a non-exercise study, Wendland *et al* (2001) recently compared lipid peroxidation and plasma antioxidant vitamin concentrations between thirty-seven (n = 37) patients with Crohn's disease and thirty-seven (n = 37) healthy control subjects. Resting lipid peroxidation, assessed by breath pentane and ethane and plasma F_2-isoprostane concentrations were all significantly higher in the Crohn's disease patients than in the controls. In contrast, plasma antioxidant vitamins (ascorbic acid, α and β-carotene,

lycopene and β-cryptoxanthin) were all significantly lower in the patient group than in the controls.

Only one study was found to use plasma levels of lipid hydroperoxides as opposed to MDA in order to determine exercise-induced oxidative damage in patients (Amatuni and Saferian, 1986) (*this study was published in Russian*). Briefly, blood was drawn from patients with bronchial asthma (group 1) and chronic asthmatic bronchitis (group 2) before and after physical exercise. Lipid hydroperoxide concentration increased pre- to post-exercise in both groups and was accompanied by an increase in superoxide dismutase in group 1 only. No control group was used in this study and the fact that superoxide dismutase was up-regulated in one group and not the other warrants further investigation. Studies relating to exercise-induced oxidative stress and type 1 diabetes will be reviewed later in section 2.5.6

2.3.2 – Direct evidence of oxidative stress and exercise in healthy and pathological models

2.3.2.1 - Healthy model

Following is an overview of studies that have utilised ESR spectroscopy to examine free radical generation in healthy exercising models.

Davies et al (1982)

The Davies group in 1982 was the first to study free radical activity directly in exercising rodents. It is arguably the most quoted study within the free radical literature.

Thirty days following birth, six rats (n = 6) were fed 21 IU Vitamin E (E/kg) (control group) while six (n = 6) were fed less than 1 IU Vitamin E (deficient group). After 6 months, the rats were subjected to two different protocols using a motorised rodent treadmill.

Protocol 1: a progressive sub-maximal endurance test in which time to exhaustion was measured.

Protocol 2: a progressive intensity exercise test designed to elicit maximum work capacity of the animals.

Gastrocnemius, soleus and plantaris muscle from the lower leg, whole liver and liver homogenates were obtained post-exercise and subsequently scanned at room temperature for direct free radical activity using a Varian E 109 EPR spectrometer. In order to help eliminate the possibility of obtaining artefactual ESR results, the authors examined spectra from washed liver preparations as well as tissue homogenates. Indirect free radical activity was assessed in both muscle and liver homogenates by the thiobarbituric acid (TBARS) method. Various oxidative enzymes (succinate, malate, pyruvate, glutamate) were used in the determination of basal respiration.

The authors either failed to measure or to include in the paper results from whole muscle samples. The ESR results demonstrate a two to three-fold increase in signal intensity in muscle and liver homogenates following exercise to exhaustion. However, despite the display of respiratory data, no ESR results for the progressive incremental exercise test were incorporated.

At rest, signal height was greater in homogenates from vitamin E deficient rats, as opposed to vitamin E fed rats. This finding supports the notion that free radicals are generated under resting metabolic conditions, and that vitamin E acts as a free radical quencher. However, what seems surprising is the post-exercise ESR signals in the control group is somewhat higher than in the vitamin E deficient group, which suggests that vitamin E is not a particularly good chain-breaking antioxidant in exercising conditions, or perhaps the vitamin E deficient rats were not able to exercise sufficiently to generate oxidants.

In whole and homogenate liver samples, differences in amplitude of the ESR signal were observed between resting and exercised exhausted animals, and between control and vitamin E deficient animals. It is hypothesised that either the exercise *per se* or vitamin E

deficiency may have increased free radical concentration. Additionally, the authors speculate that increased exercise intensity could lead to faster rates of mitochondrial ubisemiquinone turnover and haemoglobin auto-oxidation leading to an augmentation in superoxide production. Although the authors display convincing homogenate spectra, one must consider the possibility of creating artefactual signals as a result of damage during homogenate preparation (Davies and Timmins, 1996).

Both exercise and vitamin E deficiency increased lipid peroxidation as indicated by TBARS production (*table 2.1*). Vitamin E deficient rats exhibit higher resting levels of malondialdehyde in both liver and muscle homogenates. Notably, the change in lipid peroxidation observed within and between groups was accompanied by an analogous change in free radical concentration. This suggests that polyunsaturated fatty acid breakdown is free radical dependant. Also reported was a decrease in mitochondrial respiratory control, suggesting inner mitochondrial membrane leakiness and decreased energy coupling efficiency.

Surprisingly, the post-exercise increase in lipid peroxidation was greater in the muscle homogenates of rats in the vitamin E fed rats in comparison to the vitamin E deficient rats. This finding may be explained by the fact that the vitamin E deficient animals had a lower mean exercising endurance time than the control group (28.1 ± 1.2 *vs.* 46.3 ± 4.1 mins), resulting in less free radical generation.

Table 2.1 – Effect of exhaustive exercise and vitamin E deficiency on rat tissue

	VITAMIN E SUPPLEMENTED				VITAMIN E DEFICIENT			
	Muscle homogenates		Liver homogenates		Muscle homogenates		Liver homogenates	
	Rest	**Exercise**	**Rest**	**Exercise**	**Rest**	**Exercise**	**Rest**	**Exercise**
R ***Activity***	*8.0 ± 0.7*	*17.0 ± 2.4*	*8.3± 0.4*	*19.5 ± 3.4*	*10.6 ± 0.4*	*13.7 ± 1.7*	*11.9± 0.8*	*14.7 ± 1.1*
TBARS ***Activity***	*27.7± 6.9*	*50.1 ± 1.0*	*32.9± 6.0*	*77.3 ± 10.2*	*39.3 ± 6.1*	*45.1 ± 9.3*	*61.0± 7.6*	*79.8± 11.6*

Modified from Davies *et al* (1982). All values are means ± SEM. R˙, free radical activity from ESR; TBARS, thiobarbituric acid reactive substances. ESR concentrations are in arbitrary units, TBARS as nmol/g tissue.

Jackson et al (1985)

Jackson *et al* examined skeletal muscle from mice, rat and man using a varian E-3 or E-109 ESR spectrometer at 77 K. After anaesthetisation, 30 mins of contractile activity (pulses of 0.1 ms duration at 50 Hz and 70 V for 0.5 s repeated every 2 secs, continuing for 30 mins) was applied to gastrocnemius muscle of female white mice or male Wistar rats. Solid dissected muscle samples (50 mg) were freeze-clamped and cooled in liquid nitrogen, before being placed in a liquid nitrogen ESR dewar cell for analysis. For creatine kinase determination, blood was drawn from a tail vein before, at the end and 60 mins post-stimulation. Rectus abdominus muscle samples were taken and rapidly frozen in liquid nitrogen from human patients undergoing surgery for various intestinal disorders. Muscle homogenates were examined using a flat cell placed in an ESR dewar flask with the spin traps 2-methyl-2-nitrosopropane (MNP) or α-phenyl-*tert*-butylnitrone (PBN).

One major ESR signal was observed in all intact and homogenate muscle samples measured at 77 K. A *g* value of 2.004 was ascribed to the ESR signals detected in these samples, which compares favourably to that reported previously by Davies *et al* (1982).

Additionally, ESR signals representative of a nitroxide adduct were detected in muscle homogenates that were treated with 25 mM of the spin trap MNP or PBN. All ESR samples analysed at room temperature proved undetectable.

One possible explanation for the unsuccessful detection of a signal in the aqueous samples measured at room temperature may be due to the fact that water possesses a high di-electric constant (Voet and Voet, 1995), which would favour the ability of the water molecules to strongly absorb the microwaves used to detect a magnetic field. In light of this, the authors highlight a loss of instrument sensitivity when utilising aqueous based samples. One option to overcome this problem is to use an organic solvent (*e.g.* toluene) as shown by Tortolani *et al* (1993). This method greatly improves sensitivity and provides the basis to acquire clear spectra.

The authors suggest that the radical species detected at 2.004 *g* are derived predominately from the ubiquinone region located within the electron transport chain of mitochondria. However, this has yet to be confirmed, and furthermore Swartz (1972) suggest that the *g* value may be made up of individual components, and not of single semi-ubquinone. Moreover, as rightly stated by the authors, the absence of narrow isotropic features in the spin trapped samples may indicated that high molecular weight (< 30,000 kDa) bio-polymer species such as carbon-centred radicals may dominate.

Excessive contractile activity increased venous blood creatine kinase (CK) concentration, and suggests that the muscle membrane may be under constant attack from free radical species. Additionally, the use of CK as a measure of muscle damage may be questioned, as total CK consists of three isoforms (CK_{BB}, CK_{MB}, CK_{MM}) located in regions other than muscle (Jackson, 1992).

Kumar et al (1992)

Kumar *et al* (1992) measured oxidative stress parameters in albino rat myocardium homogenates following exercise and vitamin E supplementation. Direct free radical

activity was assessed using a JEOL ESR spectrometer, while TBARS, α-tocopherol, xanthine oxidase (XO), superoxide dismutase (SOD), catalase, glutathione S-transferase (GST), selenium dependent (Se-GSH Px) and non dependent glutathione peroxidase (non-Se GSH Px) were used as supporting assays.

30 minutes of daily exercise for a period of 60 days increased free radical concentration (measured by signal peak height) in heart homogenates in the control group only. The ESR signal observed compares favourably to that previously reported by both Davies *et al* (1982) and Jackson *et al* (1985). The addition of 220 IU/kg of Vitamin E to the rat diet seemed to have attenuated any free radical production in exercising rats. This finding highlights the ability of vitamin E to act as a free radical scavenger in exercising conditions. However, the finding of Davies *et al* (1982) somewhat contradicts this finding, where they report an increase in radical production in the vitamin E supplemented group. Additionally, Kumar *et al* failed to detect an ESR signal at rest despite a high TBARS level in the control group. In support of the direct free radical measurement, tissue TBARS were significantly elevated in the exercising control group compared to the vitamin E fed animals.

Tissue SOD levels were elevated following exercise in both control and exercised rats. However, SOD was lower in the resting vitamin E group in comparison with resting controls. Se-GSH Px was found to be significantly lower in the heart of exercised control rats, with no such change in the exercised vitamin E supplemented group. A significant increase was observed in both exercising groups for non-Se GSH Px. Tissue XO concentration increased in both exercised control and vitamin E supplemented animals; however, XO levels in the supplemented groups were well below that found in both control groups. The authors conclude that exhaustive endurance exercise results in free radical mediated oxidant stress in the rat myocardium. However, despite this increase, vitamin E supplementation seems to attenuate free radical production as measured by ESR spectroscopy.

Borzone et al (1994)

Borzone *et al* (1994) used ESR spectroscopy to detect free radical activity in rat diaphragm following resistive loading. ESR spectra were detected in all groups with a 30% increase in signal amplitude observed in the experimental group. A *g* value of 2.004 was measured in all spectra, which supports findings of previous work in the area. The authors speculate that the radicals are generated from a semi-quinone type of molecule present in the mitochondria.

Somani and Arroyo (1995)

Somani and Arroyo (1995) demonstrated using PBN that nine weeks of exercise training results in free radical formation in rat heart homogenates. The hyperfine coupling constants measured from the triplet of doublets were a_N = 16.3 gauss, and $a\beta_H$ = 3.5 gauss, which have been attributed to a lipid peroxidation by-product.

Moreover, the ascorbyl radical was detected ($a\beta_H$ = 1.89 gauss) in the trained group only. The authors speculate that exercise *per se* contributes to the formation of the ascorbyl radical either by regenerating vitamin E or acting as a radical stabiliser.

Unfortunately, the authors failed to measure indirect indices of oxidative stress to support the claim that free radical production leads to peroxidative damage. As previously mentioned, Halliwell and Gutteridge (1999) claim that in order to confirm oxidative damage, two or more indirect assays must be considered.

Ashton et al (1998)

Ashton *et al* were the first to use ESR spectroscopy in conjunction with the spin trapping technique to measure directly the generation of radical species in exercising human subjects.

A threefold increase in the post-exercise ESR spectra was observed in ten (n = 10) male subjects who cycled to exhaustion (0.04 ± 0.01 pre-exercise *vs.* 0.18 ± 0.04 post-exercise, P = 0.003, arbitrary units). The hyperfine coupling constants were a_N = 13.7 gauss and $a\beta_H$ = 1.9 gauss, which indicates the radical species as being secondary lipid derived oxygen centred alkoxyl radicals. A significant increase post-exercise in ascorbyl radical concentration was also detected. The hydrogen beta splitting was $a\beta_H$ = 1.8 gauss, which compares favourably to the work presented by Somani and Arroyo (1995). Lipid peroxidation (MDA and LH) by-products increased significantly post-exercise (P = 0.0125 and P = 0.006 respectively), which proves that exercise induces oxidative stress. Although the authors used packed cell volume in an attempt to measured plasma volume shifts post-exercise, unfortunately the biochemical data were not corrected accordingly.

Ashton et al (1999)

Ashton and colleagues were the first to use ESR spectroscopy to determine the effect of vitamin C supplementation on exercise-induced oxidative stress.

The same exercise challenge was used as previously described. Results show an increase in the amplitude of the ESR signal (0.05 ± 0.02 pre-exercise *vs.* 0.19 ± 0.03 post-exercise, P = 0.002, arbitrary units) together with increased MDA (0.70 ± 0.04 $\mu mol \cdot L^{-1}$ pre-exercise *vs.* 0.80 ± 0.04 $\mu mol \cdot L^{-1}$ post-exercise, P = 0.0152) and LH (1.14 ± 0.06 $\mu mol \cdot L^{-1}$ pre-exercise *vs.* 1.62 ± 0.19 $\mu mol \cdot L^{-1}$ post-exercise, P = 0.005) in the control phase. However, after supplementation with 1000mg (*2 x 500mg tablets*) of ascorbic acid no differences were observed in ESR signal intensity (0.02 ± 0.01 *vs.* 0.04 ± 0.02, arbitrary units), in MDA (0.63 ± 0.07 $\mu mol \cdot L^{-1}$ *vs.* 0.68 ± 0.05 $\mu mol \cdot L^{-1}$), nor in LH (1.12 ± 0.21 $\mu mol \cdot L^{-1}$ *vs.* 1.12 ± 0.08 $\mu mol \cdot L^{-1}$) concentration. As expected exogenous ascorbic acid supplementation significantly increased plasma ascorbic acid concentrations (26.28 ± 5.77 $\mu mol \cdot L^{-1}$ pre-supplementation *vs.* 117.54 ± 8.96 $\mu mol \cdot L^{-1}$ post-supplemenation, P = 0.005) and total antioxidant capacity (51 ± 45.1 $\mu mol \cdot L^{-1}$ pre-supplementation *vs.* 1680 ± 36.1 $\mu mol \cdot L^{-1}$, P = 0.002). The hyperfine coupling constants and radical species were identical to that previously reported (Ashton *et al,* 1998).

A 1000mg of vitamin C supplementation attenuated resting ESR values (0.02 $\pm$ 0.01, arbitrary units) in comparison to control values (0.05 $\pm$ 0.02, arbitrary units). However, one must question the effects of abolishing resting levels of free radicals with antioxidant supplementation, since cell signalling, wound healing, mitochondrial biogenesis, and the immune response (Halliwell and Gutteridge, 1999), in addition to others are positively linked to free radical generation. The authors conclude that acute ascorbic acid supplementation attenuates exercise-induced oxidative stress.

McArdle et al (1999)

McArdle and colleagues measured free radical activity following pliometric contraction-induced injury to the extensor digitorum longus (EDL) muscles of 15 pathogen free Wistar rats. The protocol consisted of three 5 min bouts of 75 contractions (*stimulation frequency of 100 Hz every 4 s and then further stretched to the optimal fibre length*) with a recovery period of 5 min between each of the three bouts. Measurements for free radical activity 3 hrs and 3 days post-exercise were made on a Varian E3 spectrometer.

For all muscle samples at 77 K, a *g* value of 2.0036-2.0040 was observed, thus the authors identify the species as mitochondrial semiquinone, which concurs with previous reports by the same group (Jackson *et al*, 1985) and others (Davies *et al,* 1982). At 3 hrs or 3 days post-exercise no difference in the amplitude of the signal was observed between contracted muscle and non-exercised control muscle. Additionally, no PBN spin adducts from abdominal blood measured at room temperature were detected from either exercised or non-exercised animals. Total muscle and oxidised glutathione content were unchanged 3 hours after stimulation; however, muscle oxidised glutathione was elevated 3 days post-exercise. Muscle TBARS levels at 3 days following the pliometric protocol were lower in comparison with nonexercised muscle; this may be related to the adaptive repsonse of cells to stress.

The authors conclude that semiquinone type radicals are not increased following pliometric contraction in rat EDL muscle, but can be following isometric contraction in rat

gastrocnemius muscle as previously shown (Jackson *et al,* 1985). The authors also suggest that their free radical detection does not conclusively indicate that other free radicals are not involved in the muscle damage process.

Table 2.2 – Summary of studies outlining ESR spectroscopy and exercise in the healthy model

Author and Date Published	***Human or Animal Study***	***Brief Description of Findings***
Davies *et al* (1982)	Animal (Rat lower limb muscle and liver homogenates)	112% and 134% increase in both the muscle and liver homogenates respectively
Jackson *et al* (1985)	Animal/Human (Rat gastrocnemius and male rectus abdominus muscle)	Increase in amplitude of ESR signal post-stimulation
Kumar *et al* (1992)	Animal (Rat heart tissue homogenates)	Increase in amplitude of ESR signal post-exercise
Borzone *et al* (1994)	Animal (Rat diaphragm)	Increase in amplitude of ESR signal post-resistive loading
Somani and Arroyo (1995)	Animal (Rat heart tissue homogenates)	Increase in amplitude of ESR signal post-exercise
Ashton *et al* (1998)	Human (Venous blood)	350% increase in amplitude of ESR signal post-exercise
Ashton *et al* (1999)	Human (Venous blood)	280% and 100% increase in amplitude of ESR signal in control and supplemented group respectively post-exercise
McArdle *et al* (1999)	Animal (Rat extensor digitorum longus muscle)	ESR signals detected but no difference between groups

2.3.2.2 - Pathological model

ESR and its application to both exercise and disease is a recent phenomena. However, no study has yet been published concerning ESR spectroscopy and exercise in a pathological model. Therefore, a limited number of studies that have utilised ESR spectroscopy in various pathological cases will be discussed.

Lohmann et al (1979)

Blood was drawn and lyophilised from 40 Leukaemia patients before and after treatment for the assessment of room temperature ESR activity. All patients were treated daily for 4 weeks with prednisolone (inflammation inhibitor) (2mg/kg of body weight), with 2 mg vincristine (a leukaemia drug) on days 1, 8, 15, and 30, and with 30 mg of doxorubicin (a leukaemia drug) on days 17 and 30. 5 mM of $CuCl_2$ was also added to duplicate blood samples. Before treatment began, ESR results showed an increase in spin concentration and an additional peak not present in control samples. It is worth pointing out that the process of lyophilisation has been shown to produce artefactual signals (Beckley, 1976), however, the failure to detect signals in the control would therefore suggest any differences observed is due to pathology and are not artefactual signals. The ESR signal was located at 2.005 *g*, agreeing with the work of Davies *et al* (1982). The ESR spectra peak height was concomitantly reduced during treatment, achieving near control spectra of healthy volunteers with the addition of 5 mM of $CuCl_2$ to the blood samples. This gradual fall in spectra height was in parallel with the fall in leukocyte count. The authors point out that even the blood of patients with a low leukocyte count experienced a change in peak height, which would suggest that other molecular changes within the blood and not the amount of leukocytes must be responsible for the spectral changes observed.

Tortolani et al (1993)

ESR was used to determine the presence of free radical species in coronary sinus blood from patients undergoing elective open-heart surgery and cardioplegia. Coronary sinus

blood samples were taken from six (n = 6) patients (3 male and 3 female) at 1, 3, 5, 10, 15, 20, and 25 minutes post aortic cross-clamp (reperfusion) and immediately mixed with the spin trap PBN and subsequently extracted with toluene. Control samples were withdrawn prior to cross clamping. The time course of free radical production during reperfusion was similar for five of the six patients (25-min sample was uncollectible for one patient) and followed a biphasic pattern, with maximal signals detected at 5 min and 25 min and low levels detected during the initial 1-3 min. Total PBN adduct production during reperfusion increased in patients subjected to longer aortic ischemia times. All spectra recorded from the six patients displayed a characteristic triplet of doublets and were consistent with the formation of alkoxyl and carbon-centred radical adducts of PBN (a_N = 13.6 gauss, $a\beta_H$ = 1.9 gauss) as shown by others (Garlick *et al* 1987, a_N = 13.6 gauss, $a\beta_H$ = 1.56 gauss; Ashton *et al* 1998, a_N = 13.7 gauss, $a\beta_H$ = 1.9 gauss).

Delmas-Beauvieux et al (1998)

Delmas-Beauvieux and co-workers were the first to apply the use of ESR spectroscopy to type 2 diabetes. Twenty (n = 20) mixed type 2 diabetics (8 men and 12 women) with poor glycemic control (HbA_{1C} = 9 $\pm$ 0.4%), and twenty (n = 20) normoglycemic healthy subjects (7 men and 13 women) (HbA_{1C} = 5.15 $\pm$ 0.12) gave venous blood for the determination of oxidative stress parameters (ESR, MDA, vitamins E and A, glutathione, superoxide dismutase, glutathione peroxidase and catalase). 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline N-oxide (DEPMPO) was used as the spin trap and dimethyl sulfoxide (DMSO) was used as a chemical stabiliser for plasma ascorbyl free radicals (AFR). Plasma lipid peroxidation was characterised by higher levels of MDA, decreased levels of glutathione and of vitamin A in the type 2 diabetic group than in the controls. Unfortunately, the signal-to-noise ratio (9:1) of the ESR signal was too low to allow identification of significant radical DEPMPO adducts in both groups. However, a significant difference was observed in the DMSO/AFR index between controls (24.00 $\pm$ 4.10 nmol·L) and diabetes (7.28 $\pm$ 2.36 nmol·L) suggesting a decrease in the plasma ascorbate pool which may be related to the increased MDA values found in the clinical population. A typical AFR doublet with g = 2.0054 and $a\beta_H$ = 1.88 gauss was observed in all spectra. This agrees with

the work of Ashton *et al* (1998) who detected the ascorbyl free radical pre- and post-exercise with characteristic spectra of $a\beta_H$ = 1.8 gauss. No correlation was found between DMSO/AFR index and the duration of diabetes, the levels of glycemia, HbA_{1C} and any indirect parameter of oxidative stress.

Gonet et al (1999)

ESR spectra were obtained from lyophilised blood samples of 25 healthy subjects and 43 patients with ischemic heart disease (33 with experience of myocardial infarction and 10 with angina pectoris). Blood was drawn from the latter group 24 hrs and 5 days after admission to hospital. ESR signals from both groups were identically located at $g = 2.005$, agreeing with the previous work by Lohmann *et al* (1979). Paradoxically, spectra from the patient group showed a weaker signal amplitude in comparison to the healthy group. Unfortunately, the authors failed to explain the difference in findings or to measure indirect by-products of free radical damage.

Etz et al (2000)

Etz *et al* (2000) examined seven (n = 7) patients undergoing elective cardiopulmonary bypass for myocardial revascularisation. Arterial and venous blood samples mixed with PBN were obtained before, during and after extracorporeal circulation. Results show a 300% increase in the arterial blood ESR signal (compared to initial concentration) 30 mins after the start of extracorporeal circulation. 24hr after the operation, free radical concentrations decreased to that of the preoperative values. This study provides evidence that hyperoxic ($PaO_2 \geq 150$mmHg) extracorporeal circulation stimulates the generation of free radical species.

Anderson et al (2001)

This group recently published the second of only two studies concerning the use of ESR spectroscopy in type 2 diabetes. The aim of the study was to test the hypothesis that

triglyceride rich lipoproteins, produced during post-prandial lipaemia could induce oxidative stress that may lead to endothelial dysfunction. Twelve (n = 12) type 2 diabetic patients with poor glycaemic control (HbA_{1C} = 8.5%), and twelve (n =12) age matched healthy controls freely gave blood, before and 4 h after a fat test tolerance meal (consisting of a homogenised milkshake containing 80 g of saturated fat, with a calorific value of 1480 Kcal) for the determination of two oxidative stress parameters (ESR and TBARS). Endothelial function was measured using a high-resolution ultrasonic wall-tracking system. There were no differences in baseline PBN adduct concentration between groups, however, TBARS was found to be higher in the diabetic group in comparison with the control group ($P < 0.05$). TBARS and PBN adduct concentration increased post-prandially in both the control and diabetic groups, however, the PBN adduct increase was 2-fold greater in the diabetic group. Endothelial dysfunction was greater at rest in the diabetic group when compared to the control group, and significantly deteriorated post-prandially. The authors suggest that the ESR spectra are alkoxyl (a_N = 13.9 gauss, a_H = 2.2 gauss) and carbonyl (a_N = 14.1 gauss, a_H = 4.0 gauss) radicals trapped as a result of the decomposition of lipid hydroperoxides. The authors conclude by suggesting that patients with type 2 diabetes have more pronounced endothelial dysfunction in comparison to healthy controls, which may be related to the contents of a high fat meal and a greater increase in free radical production.

Table 2.3 – Summary of studies outlining ESR spectroscopy in pathological models

Author and Date	***Human or Animal Study***	***Brief Description of Findings***
Lohmann *et al* (1979)	Human (venous blood from patients with leukaemia)	Increase in ESR amplitude
Tortolani *et al* (1993)	Human (venous blood from patients undergoing elective cardioplegia)	ESR detection of oxygen- and carbon-centred radical species
Delmas-Beauvieux *et al* (1998)	Human (type 2 diabetes)	ESR detection of ascorbyl free radicals
Gonet *et al* (1999)	Human (Ischemic heart disease patients)	Patient group displayed weaker amplitude ESR signals
Etz *et al* (2000)	Human (elective cardiopulmonary bypass)	300% increase in ESR signal 30 mins after start of an operation
Anderson *et al* (2001)	Human (type 2 diabetes)	Increase in ESR signal intensity following a high fat meal

It has been suggested that ESR spectroscopy is the least ambiguous method for detecting free radical species (Bini *et al*, 1999). This statement comes in parallel with the call for a more routine method of oxidative stress measurement in clinical medicine (Pryor, 1993). The previous studies outlined have applied ESR spectroscopy to clinical research, including two studies investigating patients with type 2 diabetes. However, no study has been published examining free radicals directly using ESR spectroscopy and PBN in patients with type 1 diabetes mellitus.

2.4 – Antioxidant defence systems

Previous sections have outlined the sources and biological implications of harmful and damaging oxygen radicals, and other activated oxygen species, to which we are continually exposed. Fortunately, the body is not defenceless against the generation of ROS species. All mammalian aerobic organisms utilise a series of primary antioxidant defences in an attempt to protect against oxidative damage.

The term 'antioxidant' can be defined as "*any substance which, when present at much lower concentrations than an oxidisable substrate, significantly delays or prevents oxidation of that substrate.*"

(Halliwell and Gutteridge, 1999)

In broader terms, Sen and Hanninen (1994) outline the various physiological roles that antioxidants play in the human body:

(1) Prevention of ROS formation,
(2) Interception of ROS attack by scavenging the reactive metabolites and converting them to less reactive molecules and/or by enhancing the resistivity of sensitive biological targets to ROS attack,
(3) Avoiding the transformation of less reactive ROS (*e.g.* $O_2^{\cdot-}$) to more deleterious forms (*e.g.* $OH^{\cdot}$),
(4) Facilitating the repair of damage caused by ROS,
(5) Providing (*e.g.* as a cofactor or by acting to maintain a suitable redox status) a favourable environment for the effective functioning of other antioxidants.

The following section will review the primary enzymatic and non-enzymatic antioxidant defence mechanisms *in vivo*.

2.4.1 – Antioxidant enzymes

The discovery of superoxide dismutase (SOD) by McCord and Fridovich in 1969, provided much of the basis of our current understanding of antioxidant defence systems, thus, it is appropriate to consider SOD and its isoforms first (Halliwell and Gutteridge, 1999).

2.4.1.1 - Superoxide dismutase (SOD) and its isoforms

Total SOD represents a family of metalloenzymes that catalyses a one-electron dismutation of $O_2^{\cdot-}$ to H_2O_2, as shown in the following reaction:

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2$$

(Ji and Hollander, 2000)

SOD is known only to react with $O_2^{\cdot-}$, and does so at a very slow rate ($t½$ = 7 sec) at physiological pH (Ji and Hollander, 2000). There are four distinct forms of SOD, one of which (FeSOD) is only found in bacteria and will not be reviewed in this thesis. The other three forms are found at specific cellular locations and have a different tissue distribution within the body (Young and Woodside, 2001).

The isoform, manganese superoxide dismutase (MnSOD), is located primarily in the mitochondrial matrix of eukaryote cells and has a molecular weight of 88 000 kDa (Powers and Hamilton 1999, Ohno *et al* 1994). It contains four protein subunits, each of which consists of a single manganese atom. MnSOD has a completely different protein sequence to CuZnSOD, and is not inhibited by cyanide ions, a characteristic that separates one isoform from the other (Young and Woodside, 2001). In skeletal muscle cells, MnSOD activity accumulates to approximately 15% to 35%, whilst the remaining 65% to 85% comes from CuZnSOD (Powers and Hamilton, 1999). In contrast, Ohno *et al* (1994) estimate that 80% of the totally formed $O_2^{\cdot-}$ radicals in the mitochondria may be decomposed by MnSOD.

In contrast, copper-zinc superoxide dismutase (CuZnSOD), is located primarily in the cytoplasm of eukaryote cells and has a molecular weight of approximately 32 000 kDa (Ohno *et al,* 1994). Unlike the previous molecule, CuZnSOD has two protein subunits, each containing an active copper and zinc ion (Young and Woodside, 2001).

The isoform extracellular (EC) SOD, is found, as its name suggests, outside mammalian cells and was first studied by Marklund in 1982 (Ohno *et al,* 1994). EC-SOD is a copper and zinc-containing enzyme with high molecular weight, synthesised by few cell types, including fibroblasts and endothelial cells. It is the dominant type of SOD detectable in extracellular fluids, and is released into the systemic circulation from the endothelial cell surface. EC-SOD may therefore be involved in the regulation of vascular tone, since superoxide can interact with nitric oxide in the plasma (Young and Woodside, 2001). Moreover, there have been no reports examining the effect of exercise on EC-SOD activity (Ohno *et al,* 1994).

2.4.1.2 – Catalase

Catalase contains four protein subunits (each processing a ferric haem group), and is found in high concentrations in the peroxisomes of liver and erythrocytes, but is low in heart, brain and skeletal muscle tissue (Halliwell and Gutteridge, 1999). It is involved in the decomposition of H_2O_2 (*equation below*) and inhibits the formation of other toxic free radical molecules (*e.g.* $OH^{\cdot}$).

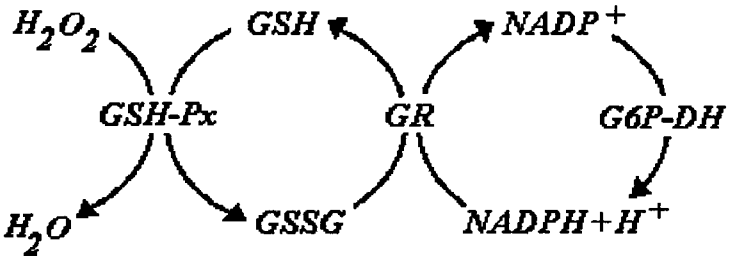
$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

Under normal conditions, H_2O_2 concentration is relatively low in the body and is efficiently removed by glutathione peroxidase, however, as the concentration of H_2O_2 increases, catalase may become more important (Halliwell and Gutteridge 1999, Brown 1993).

2.4.1.3 – Glutathione peroxidase (GSH-Px)

GSH-Px is a low-molecular mass thiol compound found intracellularly in most mammalian cells particularly erythrocytes (Halliwell and Gutteridge, 1999). Its main function is to detoxify H_2O_2 and organic hydroperoxides; and it does so by oxidising reduced glutathione (GSH) to oxidised glutathione (GSSG). The ratio of reduced to oxidised glutathione is usually kept high and constant by the enzyme glutathione reductase and NADPH (Halliwell and Gutteridge, 1999). The $NADP^+$ is further reduced by the enzyme glucose-6-phosphate dehydrogenase (*figure 2.7*), provided by the pentose phosphate pathway (Powers and Hamilton 1999, Brown 1993, Young and Woodside 2001). However, in skeletal muscle NADPH is primarily produced by isocitrate dehydrogenase (Powers and Hamilton, 1999). It is thought that GSH-Px is more important than catalase in H_2O_2 detoxification, perhaps due to the fact that it is located near SOD (Halliwell and Gutteridge 1999, Brown 1993).

Figure 2.7 – Principle aspects of glutathione redox metabolism



H_2O_2, hydrogen peroxide; H_2O, water; GSH-Px, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidised glutathione; GR, glutathione reducase; G6P-DH, glucose-6-phosphate dehydrogenase

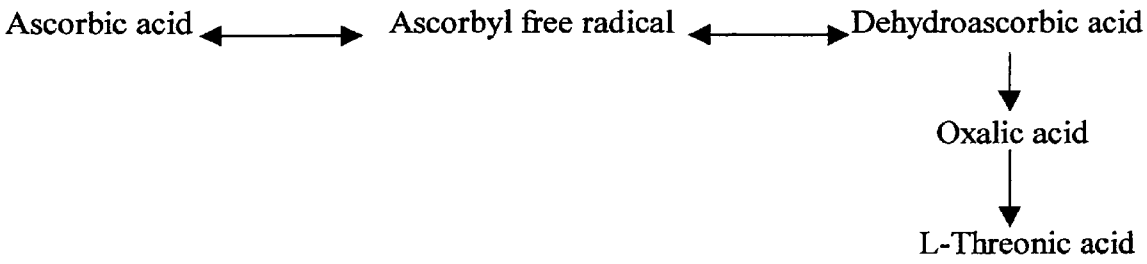
2.4.2 – Non-enzymatic antioxidants

Many important non-enzymatic antioxidants exist in cells, which include, but are not limited to ascorbic acid, α-tocopherol and the carotenoids (Powers and Hamilton, 1999).

2.4.2.1 – Ascorbic Acid

Vitamin C is the generic term for all compounds that provide the biological effect of L-ascorbic acid (Elmadfa and Koenig, 1996). Ascorbic acid is a dibasic acid, with an enediol group embedded in a five membered lactone ring. The molecular structure contains two ionising hydrogen atoms that gives the compound its acidic character. In aqueous environments, ascorbic acid is readily oxidised to the ascorbyl radical and furthur to dehydroascorbic acid, oxalic acid and L-threonic acid as shown in figure 2.8 (Elmadfa and Koenig 1996, Tsao 1997). Since, the p*K*a of ascorbic acid is 4.25, the ascorbyl radical is the dominant form existing at physiological pH (Halliwell and Gutteridge, 1999).

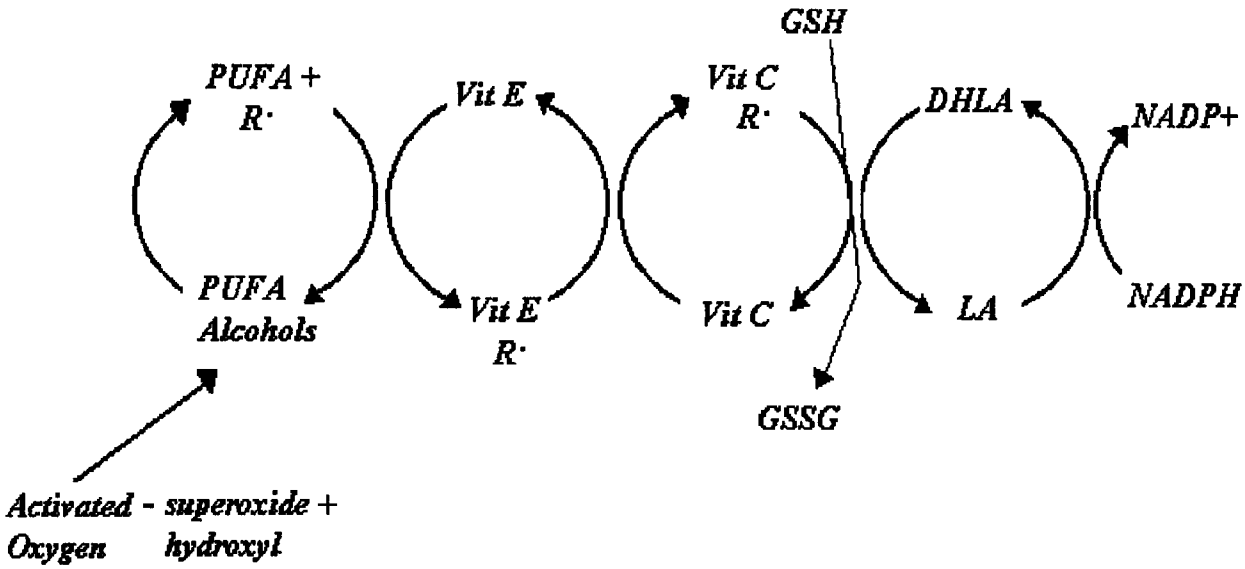
Figure 2.8 – Ascorbic acid degradation properties



Vitamin C is a hydrophilic antioxidant found in relatively high concentrations in the adrenal and pituitary glands (Powers and Hamilton, 1999). It inhibits lipid oxidation and can directly scavenge a number of free radicals including, superoxide, hydroxyl, singlet oxygen, peroxyl and alkoxyl (Halliwell and Gutteridge 1999, Bodannes and Chan 1979). However, in doing so, vitamin C can be converted to a weak free radical, which can be regenerated back to its original form by glutathione or α-lipoic acid (*figure 2.9*) (Sen and Packer, 2000). It has been documented that vitamin C acts as a first line of defence in

whole blood (Niki *et al,* 1988) and plasma (Frei *et al,* 1988) against predominately aqueous active species such as peroxyl and alkoxyl radicals (Bendich *et al,* 1986).

Figure 2.9 – Antioxidant redox cycle



PUFA, polyunsaturated fatty acids; R·, radical; Vit, vitamin; GSH, reduced glutathione; GSSG, oxidised glutathione; DHLA, dihydrolipoic acid; LA, lipoic acid.

Frei and co-workers (1989) used human blood plasma to compare the antioxidant efficacy of vitamin C with other popular biological antioxidants (α-tocopherol, urate, protein thiols and bilirubin). It was found that vitamin C completely protects plasma lipids against oxidative damage induced by peroxyl radicals. Furthermore, plasma deficient of vitamin C (with only the other antioxidants present) was shown to be extremely vulnerable to lipid oxidation. This finding is important, in that vitamin C can inhibit lipid peroxidation, despite it being a water-soluble antioxidant. Therefore, the potential for vitamin C to inactivate/scavenge lipid-derived peroxyl and alkoxyl radicals provides the justification for its use in biological systems.

A great deal of interest exists in the use of vitamin C in clinical trials with relation to cancer prevention and cardiovascular protection (Bode, 1997). Studies suggest that vitamin C protects LDL particles from oxidation, thereby decreasing the risk of atherosclerosis (Retsky *et al* 1993, Tonstad 1995). Other studies claim that vitamin C protects against many forms of cancer, including lung, stomach and cervical cancers (Block, 1991). For example, Kromhout (1987) has shown in a longitudinal study, that individuals who smoked with a low dietary vitamin C intake had a 2.8-fold increased risk of contracting lung cancer. In contrast, Fontham *et al* (1988) has demonstrated that individuals on a high intake of dietary vitamin C were less at risk of lung cancer.

However, large exogenous doses of vitamin C intake have been questioned because of its ability to act as a pro-oxidant in the presence of transition metal ions. Ascorbic acid has the ability to reduce Fe^{3+} to the Fe^{2+} state. This is important since the majority of hydroxyl radical generation comes from Fenton chemistry. An ESR study by Kadiiska *et al* (1992) has shown hydroxyl radical formation after acute copper and ascorbic acid ingestion in rat bile samples. *In vivo* studies have also shown that ascorbic acid can induce lipid peroxidation within erythrocytes membranes (Girotti *et al*, 1985) and damage DNA within isolated lymphocytes (Podmore *et al,* 1998). However, a recent review concluded that there was little evidence to suggest that vitamin C demonstrates pro-oxidant properties *in vivo* (Carr and Frei, 1999).

2.4.2.2 – α-Tocopherol

In contrast to vitamin C, vitamin E (α-tocopherol) is a lipophilic chain-breaking antioxidant and prevents lipid peroxidation in lipoproteins and biological membranes (Maxwell, 1995). It is mainly found in cell membranes, which contain lipids that are highly susceptible to free radical attack, *e.g.* polyunsaturated fatty acids (Halliwell and Gutteridge, 1999). Although having a low concentration in the phospholipid bi-layer in comparison to lipids (*e.g. ratio of vitamin E to lipids in membrane: 1: 1000 in red blood cells*), α-tocopherol has the ability to react directly with most free radicals, before they interact with fatty acids (Janero, 1991). However, if oxidation occurs, α-tocopherol inhibits chain propagation by

free radical stabilisation. The unreactive tocopherol radical formed, as a result of these reactions, is regenerated to it's original form by vitamin C (*figure 2.9*), at least *in vitro* (Machlin and Bendich 1987, Halliwell 1991, Brown 1993). Further, ESR evidence has shown the potential of vitamin C to generate α-tocopherol in plasma subjected to continuous free-radical mediated oxidative stress (Sharma and Buettner, 1993).

2.4.2.3 – Carotenoids

α- and β-carotene are important antioxidant compounds *in vivo*, and serve as precursors of vitamin A (Halliwell and Gutteridge, 1999). Both vitamins are found in tissues, membrane structures and lipoproteins, and it is believed that β-carotene in particular, complements and is protected by α-tocopherol (Strain and Benzie, 1998). They are efficient scavengers of singlet oxygen, superoxide and hydroxyl radicals and can also trap peroxyl radicals at low pO_2, with a potency as great as α-tocopherol (Burton and Ingold 1984, Yu 1994). In contrast, they can act as pro-oxidant molecules at high oxygen tensions (Strain and Benzie 1998, Burton and Ingold 1984).

2.4.3 – Antioxidants and exercise

This section will consider the published studies pertaining to antioxidants and exercise. A *brief* overview of the antioxidant enzymes and exercise will be provided since none of these enzymes were measured in any study outlined in this thesis. A larger emphasis will be placed on the non-antioxidant enzymes and exercise, particularly the literature examining ascorbic acid supplementation, exercise and free radical production. The reader is directed towards comprehensive review articles by Powers and Hamilton (1999), Ji (1995), Kanter (1995) and Powers *et al* (1999) for a more detailed account of the relationship between antioxidants and exercise.

2.4.3.1 – Antioxidant enzymes and exercise

SOD, CAT and GSH-Px provide the first line of defence against ROS generated in the mitochondria during exercise; therefore, it is inevitable that physical exercise *per se* may influence these enzymes (Ji, 1995).

In a well–controlled study, Radak *et al* (1995) examined 49 male Wistar rats following an acute exercise test to exhaustion and showed an up-regulation of both CuZnSOD and MnSOD activity in rat soleus and tibialis muscles. The CuZnSOD isoform was shown to gradually return to baseline values after 3 days post-exercise, whereas MnSOD activity and content continued to increase during the post-exercise period. GSH-Px activity was shown to increase one day after the acute exercise bout in rat soleus, but not in tibialis muscle. Likewise, Ortenblad *et al* (1997) recently studied 8 male Danish volleyball players during six bouts of 30 s of continuous jumping. Muscle biopsy data showed an increase in vastus lateralis SOD and GSH-Px activity, but not in muscle lipid peroxidation assessed by MDA content. Furthermore, Jenkins *et al* (1984) examined 20 human subjects and found there to be a linear relationship between both SOD and CAT activity and $\dot{V}O_{2\,max}$; *i.e.,* the subjects with the highest aerobic capacity had the greater levels of these enzymes compared to subjects with a significantly lower $\dot{V}O_{2\,max}$.

Not all studies have shown an increase in acute exercise-induced enzymatic activity. Both Brady *et al* (1979) and Leeuwenburg and Ji (1995), showed no changes in GSH-Px activity after exhaustive exercise. Ji (1998) suggests that these findings may be controversial and may be explained by the low oxygen demand placed on the muscle, which would inhibit an exercise-induced up-regulation of any antioxidant enzyme.

Most studies have shown no significant alterations in CAT activity following exercise (Ji 1995, Meydani and Evans 1993). In light of this observation, Ji (1998) claims this may be due to the fact that CAT is primarily located in the peroxisomes, whereas the main source of H_2O_2 during acute exercise comes from the mitochondria.

All the attention in this area has been devoted to the relationship between *exercise training* and enzymatic activity as opposed to the acute exercise response. The majority of the studies to date report increases in total SOD activity as a function of endurance training and most have been performed in rats (Leeuwenburgh *et al* 1997, Criswell *et al* 1993). Both Criswell *et al* (1993) and Laughlin *et al* (1990) have shown an up-regulation of GSH-Px in rat soleus muscle. Miyazaki *et al* (2001) recently exercised 9 untrained male subjects for 12 weeks, and found an increase in erythrocyte SOD and GSH-Px activity, with no change in CAT. Hellsten *et al* (1996) showed an increase in GSH-Px in human vastus laterialis muscle after intense cycling training. In contrast, Tiidus *et al* (1996) showed no change in total SOD, CAT or GSH-Px activity following 8 weeks of short-term, low-intensity aerobic training, which suggests that any induction in enzymatic activity is regulated by exercise intensity and training duration.

Few studies have investigated which SOD isoform is altered after endurance training, and the findings that exist are equivocal (Powers *et al*, 1999). Higuchi *et al* (1985) completed a study involving a 3 month rodent training program and showed a 37% increase in MnSOD, but no change in CuZnSOD activity, which clearly implies that mitochondria is a source of free radicals during exercise. In contrast, Leeuwenburgh *et al* (1997) reported that 10 weeks of treadmill exercise increased the CuZnSOD isoform in rat locomotor muscle. However, a recent study by Oh-Ishi *et al* (1997) has documented an up-regulation in both CuZnSOD and MnSOD isoforms in rat diaphragm after regular endurance training. Although the literature is inconsistent, Powers *et al* (1999) suggests there is accumulating evidence to state that endurance exercise training can increase both CuZnSOD and MnSOD isoforms, at least in rodent skeletal muscle.

Numerous training studies have shown no change in CAT activity (Powers *et al* 1994, Higuchi *et al* 1985, Laughlin *et al* 1990, Leeuwenburgh *et al* 1994). In fact, some studies have demonstrated a decrease in CAT activity in localised skeletal muscle tissue (Alessio and Goldfarb 1988, Leeuwenburgh *et al* 1997), a finding which is to date unexplainable (Powers *et al,* 1999).

2.4.3.2 – Non-antioxidant enzymes and exercise

Ascorbic acid and exercise

Few studies have considered the role of ascorbic acid supplementation with relation to exercise-induced oxidative stress and muscle damage. However, where ascorbic acid has been administered, workers have concentrated on athletic performance, or have used ascorbic acid alone or in combination with other antioxidants. These studies will now be reviewed.

Although recent attention has been devoted to the efficacy of vitamin E and exercise performance, vitamin C supplementation has been popular with investigators for many years (Clarkson, 1995). In a randomised placebo-controlled study, the effect of vitamin C on aerobic and anaerobic capacity was examined (Keren and Epstein, 1980). Thirty-three ($n = 33$) sedentary males were randomly given either 1000mg of vitamin C daily or placebo over a 21-day training program. Before and after training, all subjects completed a multistage fitness test and a Wingate test for the determination of aerobic and anaerobic capacity respectively. Results indicate that no beneficial effect of vitamin C was found on either exercise test after 21-days of supplementation and training. In a similar study, Gey *et al* (1970) used a double-blind vitamin C study to examine the endurance performance of 286 Air Force officers. Half of the group was assigned 1000mg of vitamin C, while the other half a placebo tablet daily for 12 weeks. Following 12 weeks of exercise training, all subjects repeated a Cooper 12-min walk-run test. Results showed no improvement in exercise performance. Gohil *et al* (1986) studied the effects of vitamin C supplementation and vitamin E deficiency on exercise performance and vitamin C tissue content in rats. After exercising to exhaustion a 33% decrease in endurance capacity was found in the supplemented/deficient rats compared to controls. However, a 38% decrease in capacity was found in the non vitamin C supplemented vitamin E deficient rats. The author's point out that vitamin C supplementation however, does not decrease the detrimental effects vitamin E deficiency has on endurance performance. Tissue vitamin C content increased in the vitamin C supplemented rats ($P < 0.05$).

Not all studies report no change in exercise performance with vitamin C supplementation. Spioch *et al* (1966) injected 500 mg of vitamin C into the venous circulation of 20 healthy males prior to five mins of physical exercise (step test). All subjects acted as their own controls by performing the step test first without vitamin C supplementation. On the vitamin C intravenous trial, oxygen consumption was reduced by 12%, oxygen debt by 40%, heart rate by 11% and a 24% improvement was observed in mechanical efficiency. However, as none of the subjects were randomised to either trial, there was a possibility of an order effect occurring. Additionally, as Gerster (1989) correctly points out, perhaps the improvement from one exercise session to the next might have been due to a training effect.

The effect of dietary vitamin C on exercise capacity and tissue levels was examined by Lang *et al* (1997). Groups of 4-8 pigs were fed standard diets of different vitamin C content (0-4g/kg diet) for 2 weeks and tested for endurance capacity on an animal treadmill. After exhaustion, freeze clamped exercise tissue was analysed for vitamin C content. Endurance capacity increased in the higher vitamin C fed pigs, but was not in parallel with tissue content post-exercise. This finding contradicts the work of Gohil *et al* (1986) and may suggest a tissue specific interaction with other antioxidants (*e.g.* vitamin E), as outlined in section 2.4.2.1.

Vitamin C may indirectly affect exercise performance by reciprocating with other physiological functions (Lukaski, 1999). For example, Peters *et al* (1993) carried out a double-blind, placebo-controlled study to determine the effect of vitamin C on the incidence of upper-respiratory-tract infection. Forty-six (n = 46) trained marathon runners where required to ingest 600 mg vitamin C daily or placebo for 21 days prior to a marathon (26.8 miles). Symptoms of upper-respiratory tract infection were monitored for 14 days after the race. Results show that the vitamin C supplemented athletes had fewer symptoms of upper respiratory tract infection than did the athletes receiving the placebo (33% *vs* 68%, $P < 0.05$). In another well-controlled more detailed study, the influence of vitamin C supplementation on the immune response to 2.5 hr of high-intensity running was determined (Nieman *et al* 1997). Twelve (n = 12) trained runners were randomised into vitamin C (1g / day for 8 days) or placebo groups. All subjects ran at 75-80% $\dot{V}O_{2\,max}$ for

2.5 hrs. In comparison to the work of Peters *et al* (1993) this study showed no effect of vitamin C supplementation on any immune metabolite following the treadmill run.

The literature pertaining to the efficacy of vitamin C on exercise performance is inconsistent, as can be seen from the studies outlined. The general consensus of a large number of well-controlled studies is that vitamin C has no ergogenic effect in persons who are not vitamin C deficient (Evans, 2000). Moreover, Gerster (1989) claims that the research to date has been clouded by badly controlled experiments, and further suggests that "*possibly, the sample sizes used in the studies may have been too small for an effect of vitamin C to be evident. It has been estimated that, statistically, a sample size of 200-300 athletes in both treatment and control groups would be needed to detect a difference of 10 s in a 400-m swim or a 1600-m run*".

In a recent cross-over randomised placebo controlled study, nine (n = 9) male university students were given 1 g of vitamin C or placebo 2 hrs prior to exercise in order to assess the effects on muscle damage and lipid peroxidation (Thompson *et al,* 2001). Each trial, separated by 14 days consisted of 90 mins of variable intensity shuttle-running over a 20 m distance (Loughborough Intermittent Shuttle Test). Results showed an increase in plasma and lymphocyte vitamin C concentration in the supplemented group post-exercise. However, a simultaneous rise in malondiadehyde, creatine kinase and aspartate aminotransferase was found in both the vitamin C supplemented and placebo controlled groups post-exercise, suggesting that short term vitamin C supplementation is ineffective in preventing muscle and lipid damage occurring during exercise. The authors suggest that the intake of vitamin C occurred at an '*inappropriate*' time to combat the rise in damage markers. This is at variance with the work of Ashton *et al* (1999), where they supplemented with 1g of vitamin C 2 hrs before exhaustive exercise and showed no change in plasma malondialdehyde concentration post-exercise. These findings would suggest that plasma malondialdehyde detection in the venous circulation is duration rather than intensity dependent.

In another study by the same group, eight (n = 8) subjects were allocated 400 mg of vitamin C daily, and eight (n = 8) a placebo for two weeks prior to unaccustomed exercise (Thompson *et al,* 2000). After 90 mins of prolonged shuttle running (Loughborough Intermittent Shuttle Test) malondialdehyde, creatine kinase and myoglobin increased above baseline values in both groups. These findings would suggest that exercise-induced lipid peroxidation and muscle damage are unaffected by two weeks of vitamin C supplementation.

In a randomised double-blind study, Goldfarb and Boyer (1994) administered either a placebo or 1000 mg of vitamin C daily for two weeks to ten (n = 10) young male subjects. Each participant ran at 80% of $\dot{V}O_{2\,max}$ for 30 mins. Vitamin C was shown to inhibit an exercise-induced increase in TBARS and lipid hydroperoxides.

In a study to examine the effect of 500mg vitamin C supplementation on the heat shock protein (HSP) response in skeletal muscle, Jackson *et al* (1999) exercised subjects at 70% $\dot{V}O_{2\,max}$ for 45 mins, supplemented for 8 weeks and repeated the exercise challenge. Lymphocytes from blood samples taken pre- and post-supplementation and treated with H_2O_2 were also studied. Muscle biopsy data showed a significant increase (455%) in HSP70 following exercise. Vitamin C alone caused HSP70 to rise by 597%, although, no further increase was observed following acute exercise. H_2O_2 treated lymphocytes recorded an increase of 1885% in HSP content, which was attenuated following vitamin C supplementation. Supplementation with 500mg vitamin C appears to suppress this important adaptive response of muscle and lymphocytes to oxidative stress.

Twenty-four (n = 16 male and n = 8 female) university students participated in one hour of box-stepping exercise in determining the relationship between vitamin supplementation (C and E) and eccentric exercise (Maxwell *et al,* 1993). The subjects were divided equally among three groups, while group A (n = 8) received a placebo, group B (n = 8) received 400 mg of vitamin C and group C (n = 8) received 400 mg of vitamin E for 21 days before and one week after exercise. Groups B and C, as expected, had higher levels of vitamin C and E respectively before exercise began. In contrast, there were no significant changes in

plasma MDA in any group following eccentric exercise. Plasma total antioxidant capacity rose significantly following exercise, representing a net efflux of antioxidants into plasma. Creatine kinase activity peaked 24 hrs after exercise, which would suggest that antioxidant supplementation has no effect on muscle damage following eccentric exercise.

In contrast to previous studies noted, Alessio *et al* (1997) showed no effect of vitamin C supplementation on exercised-induced oxidative stress. In a crossover design, 1g/day vitamin C or placebo was supplemented for 1 day and 2 weeks in human subjects. TBARS as a measure of MDA, and oxygen radical absorbance capacity (ORAC) was measured before and following 30 mins of submaximal exercise. 1 day or 2 weeks of vitamin ingestion had no affect on resting on TBARS or ORAC. However following exercise, TBARS was 12% and 33% above rest with 1 day and 2 weeks of vitamin C supplementation respectively, compared with 46% with placebo. ORAC did not change in any condition either before or following exercise and supplementation. This study concluded that exercise-induced oxidative stress was highest when subjects did not supplement with vitamin C compared to either 1 day or 2 weeks of vitamin C supplementation.

Carotenoids and exercise

There is a distinct lack of knowledge regarding the effects of α- and β-carotene supplementation on exercise-induced oxidative stress. To this authors' knowledge, only one study has been published examining β-carotene individually and exercise, while few studies have assessed β-carotene in combination with other antioxidants.

Sumida *et al* (1997) found that β-carotene administration lowered baseline urinary 8-hydroxy-deoxyguanosine excretion levels before exercise. β-carotene supplementation elevated plasma β-carotene levels, while post-exhaustive exercise levels did not change.

The effect of an antioxidant mixture on oxidant stress during exercise was examined by Viguie *et al* (1989). Twenty-three (n = 23) trained male volunteers' exercised at 65%

$\dot{V}O_{2\,max}$ at a 5% gradient following 8 weeks of supplementing with 10mg β-carotene, 800 IU vitamin E and 1g vitamin C in combination or placebo. Plasma glutathione concentrations increased following the submaximal exercise challenge, in contrast, lactate dehydrogenase and creatine kinase activity decreased following exercise. This finding contradicts the previous study of Maxwell *et al* (1993); it is possible that a single antioxidant is not as effective at preventing muscle damage as antioxidants in combination.

Kanter and co-workers (1993) investigated an antioxidant vitamin mixture on lipid peroxidation at rest and post-exercise. Twenty (n = 20) male subjects were randomly given 30 mg of β-carotene, 592 mg of vitamin E and 1000mg of vitamin C as a mixture (n =11) or a placebo (n = 9) daily for 6 weeks. The exercise test consisted of 30 min of treadmill running at 60% $\dot{V}O_{2\,max}$ followed by 5 min of running at a pace that elicited 90% of $\dot{V}O_{2\,max}$. Results show a 3- and 7-fold increase in vitamin E and β-carotene respectively after 6 weeks of supplementation. Subjects demonstrated a lower rate of pentane and MDA production at rest in the vitamin treated group. In both groups, exercise caused an increase in pentane and MDA production. However, the antioxidant mixture significantly lowered post-exercise levels of both indices. The authors conclude that antioxidants in combination serves to lower markers of lipid peroxidation at rest and following exercise but does not inhibit the exercise-induced increase in oxidative stress.

Vitamin E and exercise

Much of the work examining exercise, oxidative stress and antioxidant vitamins, seem to have used vitamin E as the antioxidant supplement of choice; perhaps due to the relationship between the frequently measured by-products of lipid peroxidation and the lipid properties of vitamin E (Ashton, 1998). Following is an overview of the literature pertaining to vitamin E and exercise-induced oxidative stress. The reader is directed towards the review article by Takanami *et al* (2000) for a more detailed account.

The studies performed to date indicate that vitamin E supplementation reduces oxidative injury and lipid peroxidation induced by physical exercise (Meydani *et al,* 1993). The

protective effect of vitamin E on exercised-induced oxidative stress in young and older adults has been studied by Meydani *et al* (1993). Twenty-one (n = 21) sedentary male subjects (n = 9, aged 22-29 yr. and n = 12, aged 55-74 yrs.) participated in a double-blind trial, which involved ingesting either 800 IU *dl*-α-tocopherol or a placebo daily for 48 days. All subjects performed three 15-min periods of eccentric downhill running at 75% of maximal heart rate at a gradient of –16%. Supplemented subjects had less urinary TBARS levels twelve days post-exercise compared to placebo. Placebo subjects tended to have higher levels of muscle conjugated dienes, thus suggesting free radical involvement. In contrast to previous work by Maxwell *et al* 1993, vitamin E seems to confer some protection against eccentric exercise-induced oxidative injury.

Simon-Schnass and Pabst (1988) reported that 400-mg of vitamin E daily attenuated lipid peroxidation during a 10-week expedition to 8611 m above sea level. The participants were randomised either to a group receiving 200 mg dl-α-tocopherolacetate (n = 6) or a placebo (n = 6) twice daily. Pentane excretion did not change in the supplemented group while the control group demonstrated values in excess of 100% higher. The authors conclude that in a condition of lower ambient pO_2, such as that at high altitude, vitamin E may help protect cell membrane integrity. However, unfortunately these authors used only one method to quantify cell membrane damage; and as previously outlined, two or more is recommended.

Sumida *et al* (1989) investigated vitamin E supplementation and exercise-induced muscle injury in humans by examining the magnitude of mitochondrial enzyme leakage after exhaustive exercise. Each subject ingested 300 mg of D-α-tocopherol acetate daily for 4 weeks before performing an incremental exercise test on a cycle ergometer. Each subject also completed an exercise test without supplementation. MDA was shown to significantly decrease post-exercise ($P < 0.05$) with vitamin E supplementation compared with no supplementation. A reduction in mitochondrial glutamic-oxaloacetic tranaminase iso-enzyme and β-glucuronidase leakage was observed following exercise in the supplemented group compared with the non-supplemented condition, which implies increased protection from muscle damage by vitamin E. However, the use of serum enzymes in muscle damage

assessment is highly variable and may be affected by factors other than physical exercise *per se* (Takanami *et al,* 2000). Therefore, their use as indicators of *true* muscle damage must be treated with caution (Takanami *et al,* 2000).

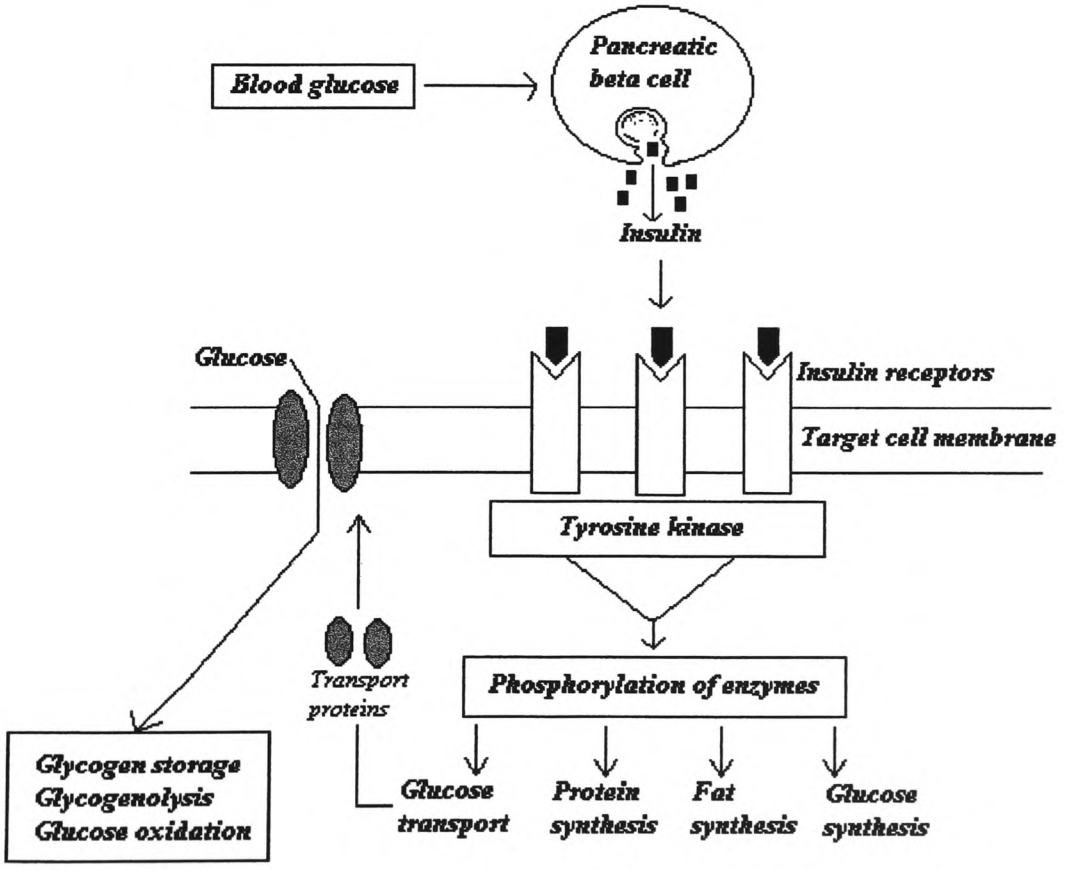
Furthermore, various studies by Warren *et al* (1992) and Helgheim *et al* (1979) fail to agree with previous work in the area, in which they show no change in serum enzyme release after exercise with vitamin E supplementation. These inconsistent reports may partially be due to the different exercise protocols used in the various studies (high-intensity *vs.* moderate-intensity exercise; concentric *vs.* eccentric muscle actions), or to the possibility that exercise-induced muscle trauma may be multifaceted, in which free radicals play only a small part in the overall damage process to skeletal muscle (Kanter, 1995). Additionally, Kagan *et al* (1994) suggest that vitamin E supplementation may benefit only those who are initially deficient.

2.5 – Diabetes mellitus

Diabetes mellitus (*mellitus* meaning '*honey-sweet*') is a chronic disease, characterised by the relative or absolute deficiency of the hormone insulin (Guyton and Hall, 2000). Insulin is a polypeptide molecule, made up of an A chain with 21 amino acids, and a B chain with 30 amino acids, held together by disulfide links (Voet and Voet, 1995). In normal biochemistry, it is produced by the Islets of Langerhans of the β-cells of the pancreas, and released into the peripheral circulation by a variety of stimuli, the most important of which, from a physiological perspective, is glucose. A specific transport protein for insulin has yet to be identified, however, it is known that insulin is transported in the plasma with the α- and β-globulins. It has a half-life of approximately 6 minutes, thus it is mainly cleared from the circulation within 10-15 minutes (Chandrasoma and Taylor 1995, Guyton and Hall 2000).

The major biochemical function of insulin is to facilitate the uptake of glucose from the plasma into the cytoplasm of target cells, such as liver, muscle and fat (Chandrasoma and Taylor, 1995). This process is regulated by the binding of insulin to a membrane receptor protein, which activates second messengers (*e.g.* tyrosine kinase), causing a cascade of cell phosphorylation that increases or decreases the activity of enzymes that mediate the effects of glucose on glucose, fat and protein metabolism (Chandrasoma and Taylor 1995, Guyton and Hall 2000). For example, glucose transporters (protein GLUT molecules) are directed to the plasma membrane to facilitate glucose entry into the cell (*figure 2.10*) (Hargreaves, 1995).

Figure 2.10 – Mechanism of insulin action on target cell and its principal biochemical actions



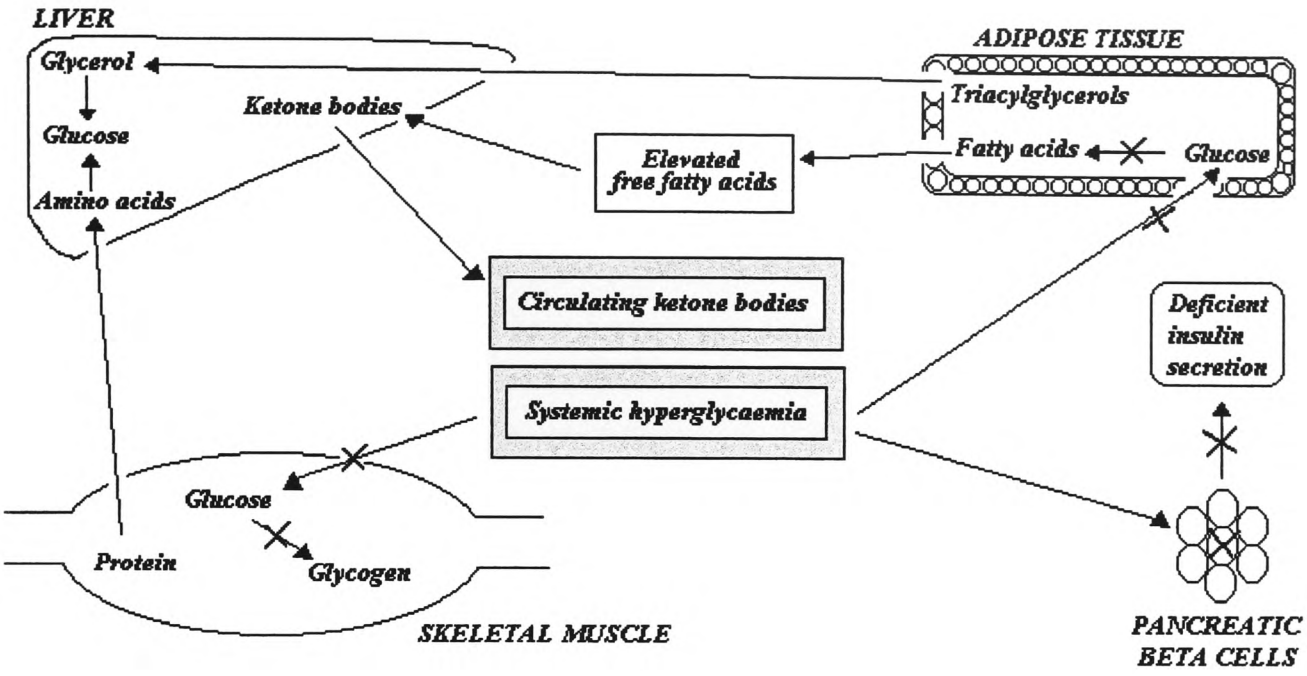
Modified from Guyton and Hall (2000)

However, in individuals suffering from diabetes mellitus, glucose entry into the cell is impaired, which can raise systemic blood glucose levels from approximately 5 $mmol{\cdot}L^{-1}$ to 40 $mmol{\cdot}L^{-1}$ (Guyton and Hall 2000, Frayn 1999). A high blood glucose level is often termed '*hyperglycaemia*'; and if left untreated can cause several alterations to metabolism (Frayn, 1999).

One of the most prominent features of hyperglycaemia, is the catabolism of other biochemical substrates (*i.e.* fatty acids from adipose tissue) (Taylor and Agius, 1988). In persons with complete insulin deficiency (type 1 diabetes), rapid mobilisation of fatty acids from adipose occurs, in addition to an inactivation of adipose tissue lipoprotein lipase (Frayn, 1999). Therefore, the failure of the adipocytes to take up excess fatty acids from the circulation causes the liver to convert the fatty acids to ketone bodies (β-hydroxybutyric acid, acetoacetic acid and acetone) (*figure 2.11*) (McCowan and Smith, 1998). In nondiabetic individuals ketone bodies are metabolised as a source of energy, however, in diabetic patients, ketone body utilisation is inhibited. An accumulation of ketones, in excess of the blood buffering capacity can lead to systemic acidaemia, causing a serious condition known *as diabetic ketoacidosis* (Taylor and Agius 1988, Frayn 1999, McCowan and Smith 1998, Randle *et al* 1963).

Paradoxically, hyperglycaemia in uncontrolled diabetic sufferers is known to increase in a fasted state. This is due to the body's natural defence mechanism counteracting the onset of low blood sugar (hypoglycaemia) (McCowan and Smith, 1998). Proteins and triacylglycerols offer amino acids and glycerol respectively to the liver, where they are converted to glucose in a process called '*gluconeogenesis*' (Frayn 1999, McCowan and Smith 1998). Thus, hepatic glucose production increases in systemic hyperglycaemia (*figure 2.11*) (Frayn 1999, Randle *et al* 1963).

Figure 2.11 – Metabolic events in uncontrolled diabetes mellitus



Modified from McCowan and Smith (1998)

The following sections will briefly review the various forms of diabetes mellitus and will discuss the relationship between oxidative stress and type 1 diabetes.

2.5.1 - Type 1

Approximately 10-20% of the diabetic population have type 1 diabetes (or often termed insulin-dependent diabetes (IDDM) or juvenile-onset diabetes) (Guyton and Hall, 2000). Type 1 diabetes is due to the destruction of pancreatic beta cells of the islets of langerhans, causing a complete insulin deficiency (Banting and Best, 1922). This lack of insulin, increases blood glucose concentration which can have multiple adverse effects on normal biochemistry, for example, polyuria, polydipsia, to more severe problems like renal failure, hypertension, atherosclerosis, cataract development, and endothelial dysfunction (*section 2.5.4*) (Guyton and Hall 2000, McCowan and Smith 1998). However, these abnormalities

may be treated or prevented by the administration of exogenous insulin. Type 1 diabetes is caused by a combination of environmental factors and genetic susceptibility, although these aetiologies are poorly understood (Guyton and Hall 2000, McCowan and Smith 1998, American Diabetic Association (ADA) expert committee report 1999).

The genetic predisposition for type 1 diabetes is associated with the inheritance of genes in the Human Lymphocyte Antigen (HLA) System (also known as the major histocompatibility complex {MHC}) (Mera 1997, Hitman 1998). Two genes within the HLA-DQ group of class II antigens have been identified as determining susceptibility for type 1 diabetes. These genes regulate the interaction between antigens and cells, and have the ability to prompt an immune response when required (Chandrasoma and Taylor 1995, Mera 1997). It is believed that certain HLA-DQ genes protect against the onset of diabetes in some individuals but not in others, while HLA-DR3 and HLA-DR4 genes appear to confer an increased risk for development of type 1 diabetes (Mera, 1997). Other genes outside the HLA system, particularly the insulin gene on chromosome 11, are implicated in the predisposition for diabetes (Mera, 1997).

The cause of β cell destruction in type 1 diabetes is largely unknown, however, autoimmunity is thought to be the main mechanism involved (Chandrasoma and Taylor, 1995). This process occurs when a viral infection is located near the pancreatic β cells, and the body mounts an immune response to the foreign antigen (Chandrasoma and Taylor 1995). However, if the structure of the antigen is similar to that of the β cell, the immune response may recognise and destroy the β cells as well as the invading organism (Mera, 1997). The immune response genes linked to the HLA system may explain the genetic susceptible to type 1 diabetes (Chandrasoma and Taylor 1995, Mera 1997). Furthermore, oxygen-free radicals are generated in type 1 diabetes, and are thought to be significant in pancreatic β cell destruction, which may be accelerated as a result of low β cell protective substances (Oberley, 1988).

2.5.2 – Type 2

Type 2 diabetes, or often termed non-insulin dependent diabetes (NIDDM) or adult onset diabetes is the most prevalent form of diabetes in modern society (approximately 80-90%) (Guyton and Hall, 2000). The onset of this type is strongly associated with obesity and genetic predisposition, and is characterised by high blood glucose levels due primarily to insulin resistance at the cellular level and relative insulin insufficiency (McCowan and Smith 1998, Chandrasoma and Taylor 1995).

Insulin resistance is a process of insulin rejection by target tissue, and is thought to be caused by the defective binding of insulin to receptors, decreased numbers of insulin receptors on the target cell, and a possible abnormality in the series of events that follow insulin binding (Chandrasoma and Taylor, 1995). This prevention of efficient cellular glucose uptake and utilisation causes a rapid rise in blood glucose concentration and an increase in cellular fat and protein oxidation (Guyton and Hall 2000, Frayn 1999). Insulin resistance is common to several other metabolic disorders, including ischaemic heart disease, hypertension, dyslipidaemia and coagulation defects; together these disorders are known as the metabolic syndrome X (Hitman 1998, Chandrasoma and Taylor 1995).

Insulin resistance alone does not cause type 2 diabetes, it is also manifested when there is a coexisting pancreatic insulin secretory defect (McCowan and Smith 1998, ADA expert committee report 1999). In persons with type 2 diabetes, basal secretion of insulin is often normal; however, the rapid rise of insulin expected after a high glucose insult is greatly impaired, resulting in a high blood glucose concentration (Chandrasoma and Taylor, 1995). Endogenous insulin secretion may be stimulated by oral drugs such as sulfonylureas, therefore, unlike type I diabetes, exogenous insulin treatment is not necessary (Chandrasoma and Taylor, 1995).

The mechanism of defective insulin secretion by the pancreas is highly complex and is probably controlled by the many genes (insulin gene, glucokinase, P13-kinase, hepatic nuclear factor 4α and others) associated with type 2 diabetes (Hitman, 1998). Furthermore,

unlike type 1 diabetes, this form of diabetes is certain not to occur from an autoimmune destruction of the pancreatic β-cells (ADA expert committee report, 1999).

2.5.3 – Other types

In addition to the two most prevalent types of diabetes previously outlined, another type, termed "gestational diabetes" exists. Gestational diabetes mellitus (GDM) may be defined as *any degree of glucose intolerance with onset or first recognition during pregnancy* (McCowan and Smith, 1998). This condition is usually caused by a disruption in hormonal balance at the beginning of pregnancy (McCowan and Smith, 1998). Females who are genetically predisposed to diabetes are particularly at risk of this condition, however, the condition is reversible (McCowan and Smith 1998, Chandrasoma and Taylor 1995).

Other pathological conditions may fall under the broad term of diabetes mellitus; including destructive pancreatic disease, chronic pancreatitis, haemochromatosis, and many forms of endocrine disease, *e.g.* cushing's syndrome (cortisol), hyperthyroidism (thyroxine) and glucagonoma (glucagon) (Chandrasoma and Taylor, 1995).

2.5.4 – Complications of diabetes mellitus

Diabetic complications in most cases are caused by poor glycemic control (ADA expert committee report, 1999). Complications of diabetes may be separated into two categories: (1) macrovascular or 'large vessel' disease and (2) microvascular or 'small vessel' disease (Thomas 1998, Frayn 1999).

Macrovascular disease, affecting both diabetic and non-diabetic individuals alike, stems from the build-up of atheromatous plaque in large blood vessels, which may partially or completely block the vessels of the heart and brain (Thomas, 1998). This impairment can cause myocardial infarction and cerebral arterial occlusion (stroke); two of the most prevalent causes of death in diabetes (Thomas 1998, Chandrasoma and Taylor 1995). Abnormal blood lipid profiles (hypertriglyceridemia and hypercholesterolemia) in diabetes

is regarded as a contributing factor in the development of atherosclerosis (Chandrasoma and Taylor, 1995).

Kidney (nephropathy), nerve (neuropathy), and eye (retinopathy) damage, are all common problems associated with microvascular disease in diabetes (Thomas, 1998). In general, these problems are caused by the binding of blood sugar to blood proteins such as haemoglobin and albumin (glycosylated), which, when transported around the microvascular circulation can cause damage to capillary walls, eventually leading to the complications associated with the disease process (Dodson, 1998). This association has been confirmed in a large scale American study, that showed when blood glucose was kept within a normal range (between 3.9 to 6.7 $mmol{\cdot}L^{-1}$), thus improving glycemic control, diabetic complications were significantly reduced (Diabetic Control and Complications Trial {DCCT}, 1993).

Oxidative stress mechanisms have also been implicated in the pathogenesis of diabetic complications (Baynes and Thorpe, 1999) which will be reviewed in the following section.

2.5.5 – Reactive oxygen species in the pathogenesis of type 1 diabetes

The first evidence that oxidative stress might be increased in diabetes mellitus appeared in 1979, when Sato *et al* reported an increase in TBARS in the serum of diabetic patients (Young, 1994). Since then, the link between diabetes and oxidative stress has been extensively investigated (Halliwell and Gutteridge, 1999). There are a number of biochemical reasons why diabetic patients may be more susceptible to increased free radical production, these include; increased availability of transition metal ions, auto-oxidation of glucose (hyperglycaemia), production of advanced glycosylation end products (AGEs), change in the glutathione redox status, elevated plasma lipids and a decreased antioxidant defence system (Grunewald *et al* 1993, Halliwell and Gutteridge 1999, Young 1994, Oberley 1988, Villa-Caballero *et al* 2000).

Most of the studies in relation to type 1 diabetes have focused on the by-products of lipid peroxidation as an index measure of free radical production.

Sato *et al* (1979) measured lipid peroxides in the plasma of 110 diabetic patients (n = 58 men and n = 52 women) and in 331 healthy controls (n = 262 men and n = 69 women). Lipid peroxidation was increased in diabetic subjects, although when subgroups were analysed the significant increase was limited to patients with evidence of micro- or macro-angiopathy. The lipid peroxide level of poorly controlled diabetics was higher than in well-controlled diabetics. However, no significant correlation between lipid peroxide and glucose tolerance (50g glucose load) was found. No correlation was observed between lipid peroxides and triglyceride or cholesterol levels. In a follow up study (Nishigaki *et al*, 1981), the same group confirmed increased lipid peroxidation in 31 badly controlled diabetic individuals with angiopathy. The increase in peroxidation was shown to be restricted to HDL cholesterol, with no increase in peroxides in either LDL or VLDL subfractions. Santini *et al* (1997) measured lipid hydroperoxides and conjugated dienes in the blood of 72 well controlled type 1 patients and compared with 45 control subjects. Compared with controls, diabetic patients had higher lipid hydroperoxide and conjugated diene levels. No correlation was observed between any measure of oxidative stress and metabolic control (HbA_{1c}) or between cholesterol or triglyceride levels.

In a study involving children with type 1 diabetes and adolescent patients (n = 54), MDA was found to be higher than in control subjects (n = 60) (Dominguez *et al*, 1998). Plasma levels of MDA were also higher as the disease progressed. No correlation was found between MDA and total cholesterol or triglycerides. MDA was also measured as TBARS in 117 diabetic patients (n = 57 type 1, and n = 60 type 2) and 53 controls (Gallou *et al*, 1993). Results demonstrate a significantly higher TBARS concentration in both type 1 and 2 diabetic patients than in the control group. TBARS levels did not differ between diabetic types. There was no correlation found between TBARS and glycaemic control or fasting blood glucose. Although total cholesterol and triglyceride levels were reported, no correlation with MDA was made.

The relationship among circulating lipids, glycated haemoglobin (HbA_{1c}) and TBARS concentration was determined in 77 patients with type 1 diabetes and in 62 control subjects (Griesmacher *et al*, 1995). Compared with control subjects, serum TBARS was found to be 70% higher in diabetic patients. However, no change was found between groups when TBARS was normalised with total lipid content (cholesterol and triglyceride). Patients with good metabolic control ($HbA_{1c} < 6.5\%$) had significantly lower TBARS than patients with poor metabolic control. However, no significant linear correlation between TBARS and HbA_{1c} was found.

Lipid peroxidation is also elevated in erythrocyte membranes in diabetic patients. Jain *et al* (1989) found increased membrane lipid peroxidation in fresh erythrocytes from subjects with type 1 diabetes and demonstrated a positive correlation with glycated haemoglobin.

Urinary 8-iso-Prostaglandin $F_2\alpha$ (8-iso-$PGF_2\alpha$), a specific marker of lipid peroxidation, was measured in 85 diabetic patients (n = 23 type 1, and n = 62 type 2) and in 85 age-matched healthy control subjects (Davi *et al*, 1999). Of the 85 diabetic subjects, 72% had hypercholesterolemia. Urinary 8-iso-$PGF_2\alpha$ excretion was significantly higher in both diabetic groups than in the control group. There was no difference in 8-iso-$PGF_2\alpha$ levels when a comparison was made between the hypercholesterolemic and normocholesterolemic patients. Improved metabolic control was associated with a significant reduction in 8-iso-$PGF_2\alpha$ by 32% in 21 type 2 patients. Furthermore, vitamin E supplementation (600 mg/d for 14 days) reduced urinary 8-iso-$PGF_2\alpha$ by 37% in 10 type 2 patients.

In contrast to the previous studies outlined, TBARS were not found to be significantly different between twenty (n = 20) type 1 patients with good metabolic control and 10 control subjects (Ghiselli *et al*, 1992).

In a novel approach, Ceriello *et al* (1991) used ferricytochrome C reduction as a measure of superoxide anion generation in the serum of 10 type 1 diabetic patients and 10 control subjects. The amount of superoxide was significantly increased in diabetic serum and correlated with fasting plasma glucose and HbA_{1c} in both diabetic and control subjects.

After one month of improved metabolic control, superoxide production was decreased in the diabetic patients. The authors argue that glucose auto-oxidation and glycosylated proteins were the main sources of superoxide production.

Although lipid peroxidation is a frequently used index of oxidative stress measurement, the importance of lipid peroxidation in diabetes should be considered. Halliwell and Gutteridge (1999), state that in most (but not all) human diseases, free radicals are formed in increased quantities particularly as a consequence of tissue injury. Thus, their presence in bodily fluids does not provide sole evidence of a significant contribution to the disease pathology. It is therefore argued, that the presence of lipid peroxidation in diabetes is likely to be a result of tissue damage rather than a cause (Lyons 1991, Young 1994), hence oxidant formation may be of little or no consequence to pathological progression (Halliwell and Gutteridge 1999, Halliwell 1987). However, lipid peroxidation is known as an ongoing cascade of events, where intermediary or secondary free radicals (peroxyl and alkoxyl) are generated, which may contribute to further tissue or molecular damage, thus encouraging pathological progress in the onset of diabetic complications (Baynes 1991, Baynes and Thorpe 1999).

In order to fully answer the important question of *how important is oxidative stress in diabetes?* Halliwell and Gutteridge (1999), indicate that there is a need to apply modern methods of oxidative stress assessment (One modern technique is the application of ESR spectroscopy). And only when sophisticated methods are more extensively applied, will it be possible to answer this, and other key questions such as:

(1) Are all patients with diabetes more prone to oxidative stress, or only those patients with complications?
(2) Can oxidative stress in diabetes be decreased by antioxidant therapy?
(3) Is ascorbate a beneficial antioxidant in diabetes?

(Halliwell and Gutteridge, 1999)

2.5.6 – Exercise and oxidative stress in type 1 diabetes

To this authors' knowledge, there have been only two studies that have examined oxidative stress in relation to exercise in diabetes mellitus.

Laaksonen *et al* (1996) first published data concerning exercise-induced oxidative stress in young well-controlled insulin-dependent diabetic men. After a 5-min warm-up, nine (n = 9) male type 1 diabetic subjects and thirteen (n =13) matched controls (for age, body mass index and $\dot{V}O_{2\,max}$) exercised on a bicycle ergometer for 40 mins at 60% $\dot{V}O_{2\,max}$. Blood samples were drawn at rest and immediately post exercise. Plasma TBARS levels and changes in the glutathione redox status were used as confirmation of resting and exercise-induced oxidative stress. Resting plasma TBARS and total glutathione levels were elevated in diabetic patients (2.2 $\pm$0.7 *vs.* 0.9 $\pm$ 0.4 μmol.L^{-1}, $P < 0.05$; and 1,203 $\pm$ 221 *vs.* 936 $\pm$ 156 μmol.L^{-1}, $P < 0.05$ respectively). Resting plasma TBARS levels were found to be inversely correlated with $\dot{V}O_{2\,max}$ in the diabetic group (r = -82, $P < 0.05$), suggesting that trained diabetic patients have lower oxidative stress at rest. Exercise increased plasma TBARS and glutathione disulfide by 50% in the diabetic group ($P < 0.05$). Post-exercise plasma TBARS did not correlate with $\dot{V}O_{2\,max}$. A positive correlation between the relative TBARS increase in response to exercise/oxygen uptake was found in the diabetic subjects (r = 0.81, $P < 0.05$).

Another article by the same group (Atalay *et al* 1997), report an extension of data from the same study previously outlined (Laaksonen *et al,* 1996). This paper was concerned with the differences in erythrocyte CuZn-Superoxide dismutase, catalase, glutathione peroxidase and reductase activity as a function of pathology and exercise. TBARS levels were also reported, and the data are the same as previously outlined (Laaksonen *et al*, 1996). Results show a 15% higher red cell glutathione reductase activity in the diabetic group ($P < 0.05$) at rest. However, red cell CuZn-Superoxide dismutase and catalase levels were lower in the diabetic group in comparison to controls ($P < 0.05$). Following 40 mins of exercise, glutathione peroxidase increased by 14% in the control only ($P < 0.05$). Exercise did not

alter other enzymes in either group. This paper concludes by suggesting that lower antioxidant defences contribute to increased resting oxidative stress levels.

Halliwell and Gutteridge (1999) propose that exercise can decrease plasma volume, which must be accounted for in attempts to assess the significance of changes in markers of oxidative damage in plasma. This is something that the two previous studies failed to take into consideration, hence the post-exercise TBARS data may be over-emphasised and must be viewed with caution. These misleading values can contribute to the difficulty in comparing future work, and thus, a general consensus regarding the extent of oxidative damage occurring during exercise in type 1 diabetes is difficult to ascertain.

There is a clear paucity of data concerning free radical generation and exercise-induced oxidative stress in type 1 diabetes. A limitation of the two studies previously published is that only indirect assays have been used to quantify oxidative stress. Several clinical studies to date have utilised ESR spectroscopy to examine oxidants in several pathological cases including angioplasty (Grech *et al,* 1996), cardioplegia (Tortolani *et al,* 1993) and more recently in type 2 diabetes (Anderson *et al,* 2001). However, there are currently no studies that have used ESR spectroscopy with or without exercise to examine free radical concentration in type 1 diabetes.

2.5.7 – Antioxidant defences in diabetes mellitus

There is a wealth of evidence to suggest that antioxidant defences are impaired in diabetes, particularly with relation to the important chain-breaking antioxidants and also superoxide dismutase (Young, 1994). This section will briefly outline the various antioxidant defences in the pathogenesis of diabetes mellitus. Where appropriate, studies concerning antioxidant supplementation will be reviewed.

Antioxidant enzymes

The majority of studies report decreased SOD activity in the presence of diabetes (Young, 1994). Loven *et al* (1986) report a decrease in Cu-ZnSOD in liver, kidney and erythrocytes of diabetic rats. However, both oral glutathione administration and intramuscular insulin was shown to reinstate SOD activity in the liver and kidneys but not in the diabetic erythrocytes. Skrha *et al* (1996) also observed lower CuZnSOD activity in 47 type 1 diabetic patients, whilst Kawamura *et al* (1992) showed that red cell CuZnSOD can become glycated in the presence of glucose, causing a reduction in enzymatic activity. Extracellular glycated SOD has been shown to be higher in diabetes (Adachi *et al*, 1991), suggesting that extracellular antioxidant defences may be impaired, resulting in endothelial damage by superoxide anion (Szaleczky *et al,* 1999).

In contrast to these reports, Kaji *et al* (1985) has demonstrated that activity of SOD is unchanged in diabetes. There is no evidence that the diabetic subjects in this study had better glycemic control than those in the studies described previously, therefore, the reason for these contradictory results remain unclear (Young, 1994).

Catalase

Studies regarding catalase activity in type 1 diabetes are contradictory. Srinivasan *et al* (1997) showed decreased catalase activity in 10 borderline (*fasting blood glucose* = 115-139 mg/dl) and 30 diabetic ($\geq$ 140 mg/dl) subjects compared to 20 normal glucose tolerance (< 115 mg/dl) controls. Erythrocyte catalase activity was found not to change either in diabetic rats (Wohaieb and Godin, 1987) or in type 1 diabetic patients (Kaji *et al* 1985). Moreover, a study by Tho *et al* (1988) found no relationship between erythrocyte catalase activity and serum glucose, lipids, fructosamine or glycated haemoglobin in a mixed group of diabetic patients. However, a study by Wataa and co-workers (1986) report elevated catalase activity in erythrocytes from diabetic children.

These discrepancies may be explained by highlighting confounding factors such as the duration of diabetes, mode of treatment, presence or absence of complications, variability in the diabetes models used (animal *vs.* human) and the age of the animals at the induction of diabetes (Szaleczky *et al,* 1999).

Glutathione peroxidase

Contrasting data exist regarding glutathione peroxidase activity in diabetes. Levy *et al* (1999) observed a 30% decrease in glutathione peroxidase activity in erythrocytes of 20 patients with diabetes. Srinivasan *et al* (1997) also showed a lower concentration of glutathione peroxidase activity in patients with borderline glucose tolerance and in type 2 diabetes when compared to healthy controls. In contrast, Vijayalingam and co-workers (1996) demonstrated increased levels of red cell glutathione peroxidase in a mixed group of diabetic patients in comparison to healthy controls. Kaji *et al* (1985) reported no difference in erythrocyte glutathione peroxidase in 60 females with type 2 diabetes compared to 71 controls. No overall consensus can therefore be drawn with regard to the status of glutathione peroxidase in diabetes (Young, 1994).

Lipid chain breaking antioxidants

Vitamin E concentration has been evaluated in several studies concerning patients with diabetes, but few have corrected for lipids. This is important due to the fact that tocopherol circulates in the plasma in lipoprotein molecules (Young, 1994). Asayama *et al* (1993) report increased α-tocopherol levels in type 1 diabetic children, a result that should be viewed with caution due to the increased prevalence of hyperlipidaemias in diabetic patients (Young, 1994). Vitamin E levels were shown to be identical in nineteen (n = 19) male type 1 diabetic patients when compared to twenty (n = 20) controls, but were lowered when corrected for cholesterol (Tsai *et al,* 1994). In contrast, both Srinivasan *et al* (1997) and Vijayalingam *et al* (1996) observed decreased levels of vitamin E in the blood of diabetic patients. Unfortunately no lipid data was presented to ascertain whether the patients were normocholesterolaemic. As outlined by Young (1994), there is a clear need

for studies to correct vitamin E for lipids in order to clarify whether diabetic patients have raised, lowered or have no change in vitamin E status.

Vitamin A was shown to be significantly lower with no change in carotene in a mixed group of type 1 diabetic patients in comparison to healthy controls (Tsai *et al,* 1994). No change in vitamin A was observed in 17 newly diagnosed and 67 type 2 uncomplicated diabetic patients in comparison to a control group (Vijayalingam *et al*, 1996). In contrast, a recent study has shown that β-carotene was significantly lower in borderline diabetic patients when compared to a control group (Srinivasan *et al*, 1997).

Aqueous chain breaking antioxidants

The majority of human studies concur that vitamin C is generally lower in both type 1 and 2 diabetes. Whether the deficient levels of vitamin C are as a result or cause of the pathology has yet to be established. However, the low levels would suggest that diabetics are not well protected against oxidative damage and may therefore be more prone to oxidative damage (Bode, 1997).

Yue *et al* (1989) found plasma ascorbic acid concentrations to be decreased in 30 streptozotocin induced diabetic rats in comparison to a sex matched control group. This finding was confirmed by Sinclair *et al* (1992) who also found a decrease in plasma ascorbic acid concentration in human diabetic patients with retinopathy in comparison to control subjects. This study also reports an increase in the dehydroascorbic acid/ascorbic acid ratio of the diabetic group, suggesting that vitamin C is active in combating oxidative stress. Others report decreased plasma ascorbic acid concentrations in diabetic patients in comparison to healthy controls (Yue *et al* 1990, Srinivasan *et al* 1997).

Leukocyte ascorbic acid content is the preferred index of tissue vitamin C status (Cunningham, 1988). Chen *et al* (1983) report a significantly reduced mononuclear-ascorbic acid concentration in five (n = 5) diabetic subjects when compared to nine (n = 9) healthy nondiabetic males. An intravenous glucose bolus reduced leukocyte ascorbic acid

concentration in the healthy subjects to similar levels of the diabetic subjects. Intravenous glucose did not further lower leukocyte ascorbic acid concentration in the diabetics. This finding would suggest that ascorbic acid is competing with blood glucose for cellular uptake, and indeed this has been shown in many diabetic studies (Sinclair *et al* 1992, Yue *et al* 1990, Bode *et al* 1993).

It has been postulated that vitamin C supplementation (1 g/day) may be a safe and effective means of alleviating free radical generation and their causes in diabetes (Bode, 1997). However, Sinclair *et al* (1992) give 42 diabetic patients with (n = 22) and without (n = 20) retinopathy and 22 control subjects 1 g/day for 6 weeks and found no change in either diene conjugates or TBARS levels amongst the groups.

Among the other aqueous antioxidants presence in the blood, uric acid is compromised in type 1 diabetes, and an inverse relationship between glycaemic control and serum urate levels was found in a study by Whitehead *et al* (1992). Glutathione was also found to be lower in the plasma (Samiec *et al*, 1998) and erythrocytes (Srinivasan *et al*, 1997) of diabetic patients when compared to control groups.

2.6 – Summary of literature review

It can be seen from the literature, that there is a paucity of research in the area of exercise and oxidative stress in health and disease, particularly in hypoxia and in type 1 diabetes mellitus. With this in mind, study one will use ESR spectroscopy and lipid peroxidation to address the issue of oxidative stress induced by normobaric hypoxic exercise. This will be the first study to address the association between ESR spectroscopy and exercise in normobaric hypoxia.

Will and Byers (1996) outline the need for further studies to investigate the relationship between oxidants and antioxidants in diabetes. Study two will use ESR spectroscopy and lipid peroxidation to address the issue of exercise-induced oxidative stress in type 1 diabetes. This will be the first work to address the association between ESR spectroscopy

and exercise and the effect of ascorbic acid supplementation (*study three*) on free radical production in type 1 diabetes. Biomarkers of lipid peroxidation will also be used to assess the efficacy of acute ascorbic acid supplementation on oxidative stress in this cohort.

2.7 - Development of null hypothesis (H_O)

The following statements represent the experimental variables to be considered and analysed in this thesis. The hypothesis may be stated as follows:

1. H_O – *null hypothesis*
2. H_1 – *alternative hypothesis*

Subsequently, depending on the results of the data collected and the statistical analysis performed, it is intended to reject or hold tenable the appropriate hypothesis at the level of significance established.

Hypothesis A – Study 1:

H_O – Direct and indirect indices of free radical-mediated oxidative stress are not affected by acute normobaric hypoxia.

H_1 – Direct and indirect indices of free radical-mediated oxidative stress are affected by acute normobaric hypoxia.

Hypothesis B – Study 2:

H_O – Exercise-induced oxidative stress levels are not greater in type 1 diabetic patients than in apparently healthy controls.

H_1 - Exercise-induced oxidative stress levels are greater in type 1 diabetic patients than in apparently healthy controls.

Hypothesis C – Study 3:

H_O – Ascorbic acid supplementation has no effect on oxidative stress levels in type 1 diabetic patients.

H_1 - Ascorbic acid supplementation effects oxidative stress levels in type 1 diabetic patients.

Hypothesis D – In vitro studies:

H_O – The detected free radical species do not originate from polyunsaturated fatty acids.

H_1 – The detected free radical species do originate from polyunsaturated fatty acids.

Chapter 3

General Methodology

3.0 - INTRODUCTION

This chapter outlines the theory of Electron Spin Resonance (ESR) spectroscopy and includes a description of the procedures used in data collection. Full details of equipment used, testing procedures and statistical analyses are also included.

3.1 – Introduction to Electron Spin Resonance (ESR) spectroscopy

Electron Spin Resonance (ESR) or Electron Paramagnetic Resonance (EPR) is a physical method of observing molecules with an unpaired electron in a magnetic field (Ikeya, 1993). The term *electron paramagnetic resonance* was first introduced taking into account contributions from electron orbital in addition to spin angular momentum. The term *electron spin resonance* was introduced as the absorption is linked primarily to the electron-*spin* angular momentum (Forman and Borg 1989, Weil *et al* 1994). However, nowadays both terms are synonymous and used inter-changeably, although there is a tendency to use the term Electron Magnetic Resonance (EMR) in order to make association with nuclear magnetic resonance spectroscopy (Ashton, 1998).

ESR spectroscopy was first discovered by Zavoisky (1945), following the *classical* work of Stern and Gerlach (1921) where they showed that a beam of silver atoms passing through a relatively powerful magnetic field would split in two. Since then, the use of ESR spectroscopy has become widespread in radiation chemistry and solid-state physics where free radical concentrations tend to be high (Ikeya, 1993). The application of ESR spectroscopy to biological processes can be traced back to 1954 when Commoner and colleagues (Commoner *et al*, 1954) first applied the technique to detect free radicals in growing seeds. Its use of detecting free radical species in biological samples has, however, proved difficult due to low steady state concentrations and the transient nature of the free radicals concerned (Jackson *et al* 1985, Ashton 1998). Water, the solvent where any biological reaction occurs, also diminishes the sensitivity of the instrument, as water strongly absorbs the microwaves to block any detection of free radical species (Jackson and Johnson, 1989).

Two commonly used methods of overcoming these difficulties are freezing the biological sample, usually at 77 K (Jackson *et al*, 1985), and the use of spin trapping

agents. The latter technique, first discovered in the late 1960's by Janzen and Blackburn, has provided much of our current understanding of biologically relevant free radicals in cellular, *ex vivo*, and *in vivo* systems (Davies and Timmins, 1996). For a more detailed review of spin trapping in biological systems, the reader is directed to two review articles by Janzen (1980) and Davies and Timmins (1996).

3.2 – Theory of ESR spectroscopy

In the present thesis, only a brief overview of ESR is provided, thus the reader is directed to Weil *et al* (1994) for a greater appreciation of the fundamental principles of ESR. ESR detection of free radical species is due to the unpaired electron possessing spin (S = ½), thus having a magnetic moment (dipole) which can be aligned either in parallel to, or antiparallel to an external magnetic field (Jackson, 1999). Therefore the electron can exist in two energy levels known as the Zeeman energy levels.

3.2.1 – Zeeman energy levels

The energy level of the magnetic moment of the unpaired electron is higher for the antiparallel alignment than for the parallel alignment, and the resulting energy difference termed Zeeman splitting is dependent on the external magnetic field. These energy differences (ΔE) can be measured due to an important relationship between ΔE and the absorption of electromagnetic radiation. According to Plank's law, electromagnetic radiation will be absorbed if:

Figure 3.0 – Zeeman equation

$$\Delta E = h\nu = g\beta H$$

where;

h = Planck's constant

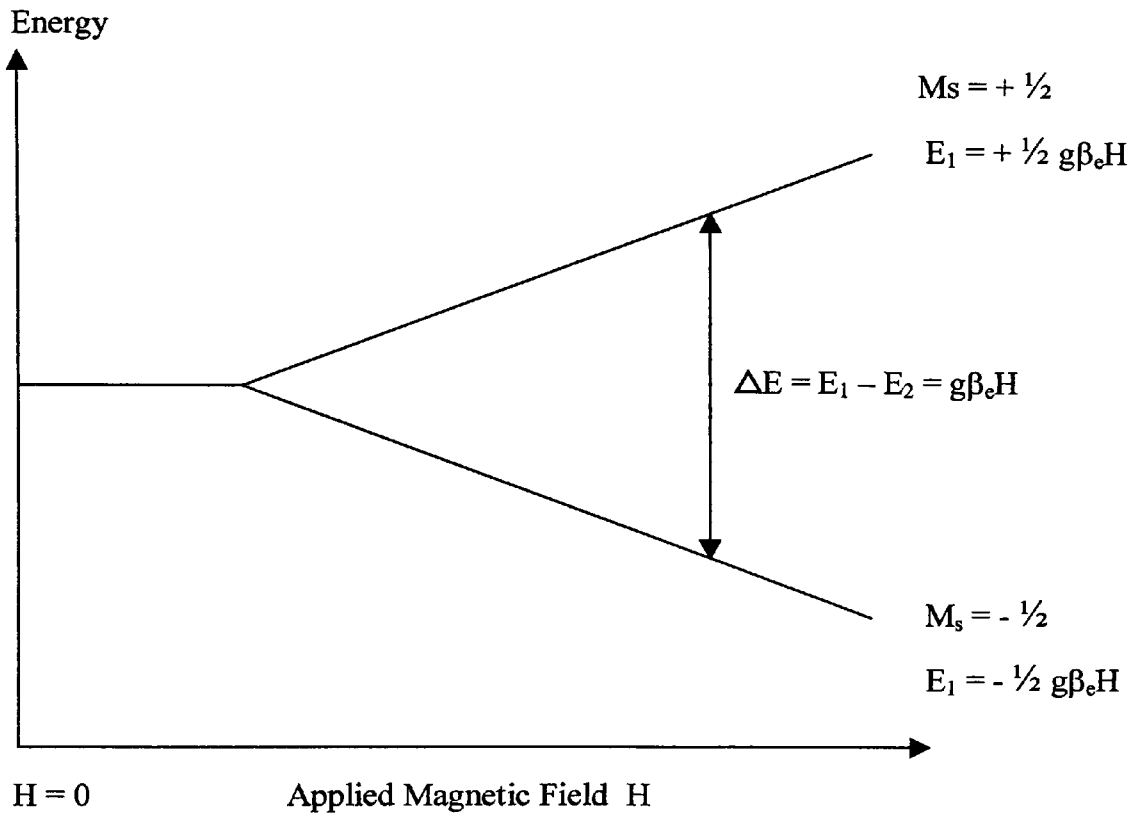
ν = frequency of electromagnetic radiation

H = Strength of external magnetic field in gauss

β = a constant known as the Bohr magneton (9.27×10^{-21} erg G^{-1})

g = spectroscopic splitting factor or g factor (g = 2.0023) (Forman and Borg, 1989)

Figure 3.1 - Zeeman energy levels of an electron in an applied magnetic field



The absorption of energy causes a transition from the lower energy state to the higher energy state as shown in figure 3.1. The two states are labelled by the projection of the electron spin, M_s, on the direction of the magnetic field. Because the electron is a spin ½ particle, the parallel state is designated as M_s (electron spin) = - ½ and the antiparallel state is M_s = + ½ (Weil *et al*, 1994). As shown in figure 3.1 the position of the absorption varies with magnetic field. Radiation frequency in ESR is in gigahertz placing it in the microwave portion of the electromagnetic spectrum, thus it is more convenient to refer to absorption with g-value reference.

$$g = \frac{\Delta E}{\beta H} = \frac{h\nu}{\beta H}$$

If the separation of the two energy levels matches the microwave energy at a particular magnetic field (for a given free radical), an absorption signal in its first derivative is recorded (Jackson, 1999).

3.2.2 - Relaxation

If electrons were to be constantly moved from a low energy to a high energy level then the population of both energy levels would equalise, resulting in the absence of radiation absorption (Ikeya, 1993). It is therefore imperative to maintain a population excess on the electron low energy state, and in order for this to occur the electrons must have the ability to transfer their excess spin energy either to other species or to the surrounding lattice as thermal energy (Ikeya 1993, Weil *et al* 1994).

The mechanism by which electrons transfer energy is known as the relaxation process, and the time for the spin system to lose one electron of its excess energy is known as the relaxation time (Weil *et al*, 1994).

3.2.3 - Signal intensity

In simplistic terms, the ESR signal intensity is proportional to the concentration of free radical species (number of electron spins) in a given biological sample, and this is due to the rapid relaxation process.

3.2.4 - Hyperfine structure

The characteristic of an ESR spectrum of free radical species in biological fluids, is its detailed pattern of peaks. ESR peaks known as nuclear hyperfine structure, originates from the interaction of the electron spin with molecular magnetic nuclei. This interaction results in the splitting of resonance lines into two or more components, so called hyperfine splitting (Forman and Borg, 1989). Nuclear hyperfine structure can aid in the identification of free radical species, as the separation and intensities of the ESR spectral lines are characteristic of the specific electron environment (Forman and Borg, 1989).

Many molecules contain nuclei consisting of a magnetic moment and thus spin angular momentum, and these can interact with the electron and spilt its energy levels to give hyperfine structure. When an uneven number of electrons interact with a single proton, the magnetic field of the proton produces a small additional field, which adds to or subtracts from the external magnetic field. This results in resonance peaks, separated by the magnetic interaction between the electron and the nucleus (Weltz and Bolton 1986, Forman and Borg 1989).

One particular component to the hyperfine interaction, is that of the fermi contact interaction. This interaction is related to the presence of an unpaired electron spin density at the nucleus, and is independent of the orientation of the free radical species in the magnetic field (*i.e.* isotropic) (Weltz and Bolton 1986, Forman and Borg 1989, Ikeya 1993).

3.2.5 – ESR spin trapping theory

Spin trapping has become a valuable tool to directly monitor free radical activity and to better understand free radical reactions in biological systems (Buettner, 1987). The general underlying principle of spin trapping is the conversion of a paramagnetic free radical by a diamagnetic component (spin trap) to a relatively stable adduct, as shown below in figure 3.2. The longer lifetime of adduct allows accumulation over time, and thus even very low rates of radical generation and/or very low steady-state concentrations can be measured by ESR spectroscopy (Davies and Timmins, 1996).

Figure 3.2 - Interaction between a diamagnetic spin trap and a paramagnetic free radical

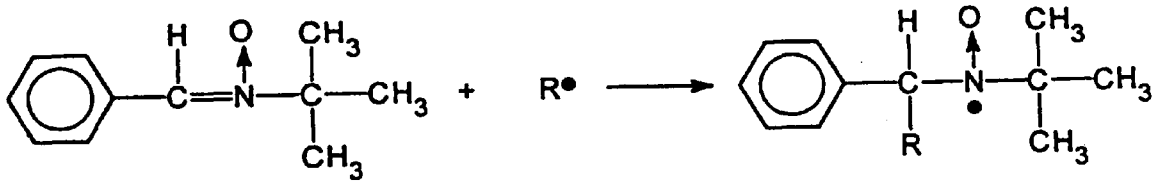
ST	+	R·	=	SA·
(diamagnetic spin trap)		(paramagnetic free radical)		(stable spin adduct)

One major advantage of the spin trapping technique is that by determination of the characteristics of the spin adduct spectrum (*i.e.* hyperfine splitting), it is possible to

identify the type of trapped radical, or at least whether the radical is oxygen, carbon, sulphur or nitrogen centred (Davies and Timmins 1996, Karlsson 1997).

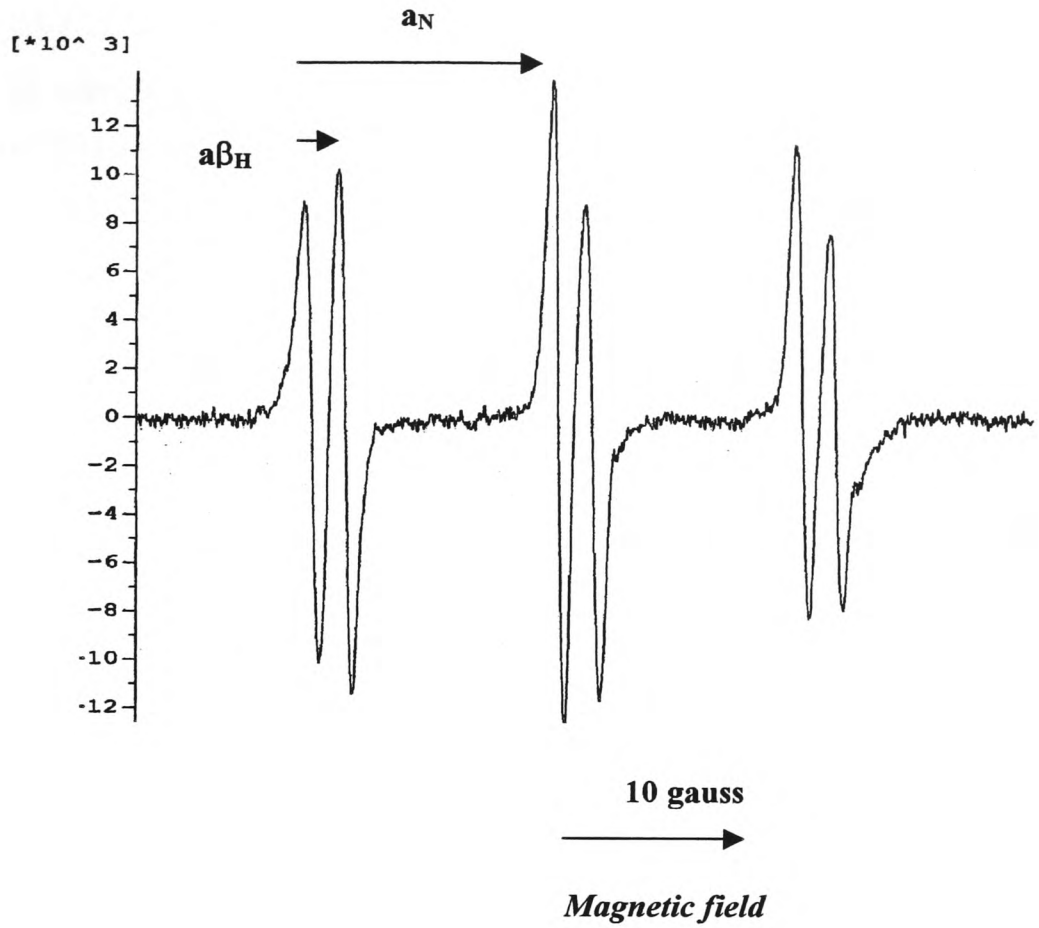
One particular category of spin traps called nitrones, consist of two commonly used spin trapping molecules, α-phenyl-*tert*-butyl-nitrone (PBN) ($C_{11}H_{15}NO$) and 1,1,5-dimethylpyrroline-N-oxide (DMPO) ($C_6H_{11}NO$) (Punchard and Kelly 1996). The application of these '*traps*' is most popular when working with biological systems as they give the longest spin-lived adducts with oxygen-centred radicals (Punchard and Kelly, 1996). However in choosing between the two spin traps, PBN appears to be the most commonly used, perhaps due the fact that it has a good shelf stability and has been commercially available for a long time (Anderson-Evans, 1979).

3.3 – The interaction between PBN and a free radical



PBN, which contains a hydrogen atom beta (β), can interact with the unpaired electron in a manor that causes each of the three nitrogen lines to be spilt into doublets resulting in a six-line spectrum. The distance between the two lines of the doublets is designated the β-hydrogen split ($a\beta_H$). The g-value (distance between the lines) which is derived from the interaction of the unpaired electron with the nitrogen is termed a_N (*figure 3.4*) (McCay and Poyer, 1989).

Figure 3.4 – ESR spectra of PBN adduct



Normally nitrone values for a_N and $a\beta_H$ are constant for a particular radical adduct in a given solvent, however, a disadvantage of PBN is that it does not distinguish between radicals since its ESR spectra generally consists of a triplet of doublets with a relatively small variation in the doublet splitting as a function of the trapped radical (Anderson-Evans, 1979).

The use of DMPO would generate comparatively more information about the radical trapped, however a criticism is its relative instability and ability to generate artefactual ESR signals (Davies and Timmins 1996, Ashton 1998).

3.2.6 – Spin adduct decay

The most common route of spin adduct decay is the reduction of the spin adduct, as illustrated in the following equation for nitroxide and the hydrogen atom:

$$-N\text{-}O^{\cdot} + H^{\cdot} \longrightarrow -N\text{-}^{\cdot}OH$$

(Anderson-Evans, 1979)

Radical adducts may also decay when two radical adducts come together to form a nitrone (Davies and Timmins, 1996). Spin adduct decay is common particularly when working with biological systems, as a plethora of potential reducing agents exist. A common reductant in biological systems is ascorbic acid, thus the rapid decay of the ESR spectral lines may well result from the presence of ascorbic acid. It is therefore unlikely that any signals detected from a biological system will be artefactually high since the presence of ascorbic acid would inhibit an increase (Ashton 1998, Anderson-Evans 1979).

To summarise, on successful detection by ESR of a spin-trapped free radical, the main priority is to identify and determine the origin of the species. However, in complex biological systems this may prove difficult as many possible mechanisms of free radical production and sites of origin exist. It is therefore recommended that supporting assays are used in addition to ESR application, as this would partially confirm the origin and identity of the ESR signal observed (Ashton, 1998). Spin trapping nevertheless, provides a powerful technique for the study of free radical production and observation by ESR in biological systems (Anderson-Evans, 1979).

3.2.7 – Spin trapping sample extraction and analysis procedure as used in the present research

1. 200mg of the spin trap α-phenyl-*tert* butylnitrone (PBN) (Sigma Ltd. Dorset, UK) was magnetically stirred with 6ml of normal saline (0.9% sodium chloride) (Baxter, UK) on each morning of an experiment. The PBN was used without further purification.
2. 1.5ml of spin trap solution was inserted into glass 6ml serum separation tubes (SST) (Becton-Dickinson Ltd. Oxford, UK). The vacutainers were kept on ice in the dark until use (approximately 10 minutes).
3. The vacutainers containing the spin trap solution were then used to take blood from an ante-cubital vein immediately pre- and post-exercise.

4. Once blood was taken, the SST containing the spin trap solution was inverted gently, and placed on ice in the dark to clot. The clotting process occurred after 10 minutes.
5. Once clotted, the SST tubes were immediately centrifuged (Centra CL3R, IEC, USA) at 3000rpm for 10 minutes at 4^0C.
6. 1 ml of HPLC grade toluene (Sigma Ltd, Dorset, UK) was added to 1ml of the serum/spin adduct and vortex (Whirlimixer, Fisons, England) mixed for 30 seconds.
7. Subsequently, the sample was centrifuged at 4000 rpm for 10 minutes to separate the organic layer.
8. 200µl of the toluene/organic layer containing the spin trap was inserted into a precision bore ESR sample tube (Wilmad Ltd, UK) and vacuum de-gassed (West Technology Ltd, UK) in a freeze (liquid nitrogen) / thaw procedure, using a turbo molecular pump (Alcatel, ACT 200T, UK) to 10^{-3} Torr for 3 consecutive 8 minute cycles. A Pirani 14 gauge detector (Edwards, APG–NW, UK) was used to monitor pressure change.
9. All samples were immediately analysed under vacuum at room temperature on a Bruker EMX series X-band ESR spectrometer (Bruker, USA, software version 3.2) under the following operating conditions: Modulation frequency, 100 kHz; microwave frequency, 9.766 GHz; incident microwave power, 20 mW; scan width, 50 gauss; modulation amplitude, 0.50 gauss; magnetic field centre, 3480 gauss; receiver gain 1.00 x 10^5; time constant, 83.9 ms; sweep time, 83.8 seconds for 10 scans.
10. All ESR samples were analysed within 1.5 hrs of blood collection.
11. Inter-assay coefficient of variation (CV) at 1795 arbitrary units = 5.2% (Davison G.W. unpublished observations).
12. All ESR spectra displayed are typical spectra are from an *n* of one.

3.3 – *Haematological measurements*

3.3.1 - *Blood sampling*

All blood sampling was carried out by the student with previous experience in the methods employed. This practice helped standardise blood handling and minimise inter-subject analytical variation. It has been shown that diet may adversely affect

several blood borne metabolites, in particular plasma lipids and lipoproteins (Pronk *et al*, 1993), thus all venous blood sampling was completed after a 12 h overnight fast. Blood was obtained from a sterilised earlobe (arterialised capillary blood) and from a forearm ante-cubital vein (venous blood). Resting capillary and venous blood samples were taken after the subject had been in a supine position approximately 20 minutes, and immediately post-exercise unless otherwise stated. This procedure in conjunction with the equations of Dill and Costill (1974) was used in an attempt to correct and control for exercise induced plasma volume shifts.

3.3.2 - *Collection of venous blood*

Each subject assumed a supine position and a tourniquet was fixed above the distal region of the subjects bicep (Bachorik, 1982). Venous blood samples were drawn after cleaning a prominent ante-cubital forearm vein with a sterilised swab saturated with 70% v/v isopropyl alcohol (Medi Swab, Smith and Nephew, UK), by using either the vacutainer™ (Becton Dickinson, Oxford, UK) or cannulation method. The latter method may be described as follows: An 18 gauge cannula (Venflon IV cannula, Becton-Dickinson, Sweden) connected to a 3 way sterile stopcock (Connecta plus 3, Ohmeda, Sweden) was inserted into a forearm vein. Blood was drawn into a vacutainer via a vacutainer holder with a luer adaptor (Becton-Dickinson, vacutainer systems, NJ, U.S.A). In order to keep the line patent, between 3-5ml of physiological saline (Norton Steri-Amp, 0.9% NaCl, Steripak Ltd, Cheshire, UK) was mixed with 0.1ml of heparin (Monoparin, 1000 units per ml, CP Pharmaceuticals Ltd, Wrexham, UK) and injected into the cannula line after each blood sample was obtained. This concentration mixture has been shown not to affect lipolysis (*Personal communication, Dr J Leiper*). After immediate blood collection, di-pottasium ethylene diamine tetra-acetic acid (EDTA) vacutainers were placed on ice, whilst the SST's were allowed to clot at room temperature before centrifugation began at 3000 rpm for 10 mins (Centra CL3R, IEC, U.S.A.). Plasma and serum were removed using a 1ml pipette (Gilson Medical Electronics, France) and transferred to 1.5 ml plastic vials (Eppendorf, Germany). Plasma and serum aliquots were stored at $^{-}70^{\circ}C$ before biochemical analysis (*within 6 months*) to minimise peroxidation during storage (Young and Trimble, 1991). Blood glucose and HbA_{1c} concentrations were analysed on collection day.

3.3.3 - *Collection of arterilised capillary blood*

The volunteer's right earlobe was wiped clean with a medi-swab. Before exercise began, the sample site was punctured using a sterile stainless steel lancet (Lance, Sheffield, UK), and subsequent blood was wiped clean with medical grade cotton wool. In order to obtain a blood sample, gentle pressure was applied to the site using the thumb and index finger. To overcome any peripheral vasoconstriction following maximal exercise, it was necessary to increase the pressure applied to the sample site to increase blood flow. Extra care was taken to remove excess sweat from the earlobe before blood sampling in order to offset diluting the sample.

3.3.4 - *Packed cell volume (PCV)*

A 75mm (59µl) heparinised capillary tube (Hawksley and Sons Limited, Sussex, UK) was used to collect arterialised capillary blood from the subject's earlobe. An air bubble free sample was sealed at the distal end with cristaseal (Hawksley and Sons Limited, Sussex, UK) and carefully inserted into a micro haematocrit centrifuge (Hawksley and Sons Limited, Sussex, UK) with the sealed end facing outwards. The capillary sample was immediately centrifuged at 11,800 revolutions per minute (RPM) for four consecutive minutes and the subsequent packed erythrocytes were measured using a Hawksley Micro Haematocrit Reader (Hawksley and Sons Limited, Sussex, UK). The value expressed in $L \cdot L^{-1}$ of whole blood was subsequently corrected by 1.5% for plasma trapped between erythrocytes (Dacie and Lewis, 1968). Duplicate samples were analysed and the mean of the two was taken as the definitive value.

3.3.5 - *Haemoglobin (Hb)*

The concentration of Hb in whole blood was measured photometrically following the method outlined by Vanzetti (1966). The principal procedure involves the release of haemoglobin from hemolysed erythrocytes by sodiumdeoxycholate. Haemoglobin is converted to methemoglobin by sodium nitrite, which together with sodiumazide forms azidemethemoglobin. The absorbance is subsequently measured at two wavelenghts (570 and 880 nm). This method has been validated against the established hemiglobincyanide (HiCN) method. Following calibration with an optical interference

filter (Hb = 13.7 $g \cdot dL^{-1}$ (8.7 $mmol \cdot L^{-1}$) $\pm$ 0.3 $g \cdot dL^{-1}$ (0.2 $mmol \cdot L^{-1}$), 10µl of arterialised capillary blood (earlobe) was accumulated in a microcuvette (HemoCue B-Haemoglobin, Sheffield, UK). The microcurvette was inserted into the photometer (HemoCue B-Haemoglobin, Sheffield, UK) and a digital result was presented in approximately 25 secs. Duplicate samples were analysed and the mean of the two was taken as the definitive value.

3.3.6 – *Lipid peroxidation assessment*

Lipid peroxidation is a repetitive process whereby PUFA molecules are degraded to a variety of end products as previously described in figure 2.6. The following techniques were employed to determine peroxidation of lipids in human blood.

3.3.6.1 – Plasma malondialdehyde (MDA)

One of the oldest and most common methods used for assessing lipid peroxidation of fatty acids in biological samples, is the thiobarbituric acid reactive substances (TBARS) assay (Janero, 1990). TBARS measures the amount of malondialdehyde (MDA) and MDA-like substances formed during the lipid peroxidation process. The technique is primarily based on the fact that, under acidic conditions, two molecules of thiobarbituric acid (TBA) will react with one molecule of MDA, producing a coloured adduct which can absorb light at a maximum wavelength of 532 nm. However, the problem with the TBARS assay is that other substances such as biliverdin, glucose ribose and 2-amino-pyrimidines also have the ability to absorb at or close to 532 nm, on heating with TBA. This assay reports higher MDA concentrations when compared with more sophisticated techiniques, and this needs to be taken into consideration when making conclusions with the exercise and oxidative stress literature. The lack of specificity within this assay may be overcome by fluorimetric detection preceded by high performance liquid chromatographic (HPLC) separation of the MDA-TBA adduct (Young and Trimble, 1991). Little if any free MDA is found in biological systems, and therefore most is derived from the breakdown of peroxides during the acid heating stage of the test (Gutteridge, 1986). In contrast, Janero (1990) claims not all lipid peroxidation products generate MDA, and MDA is produced by reactions other than lipid peroxidation. Other aldehydes such as 4-hydroxynonenal (HNE), produced as by-products of lipid

peroxidation, can be separated by gas liquid chromatography and identified by mass spectroscopy (Esterbauer *et al*, 1991).

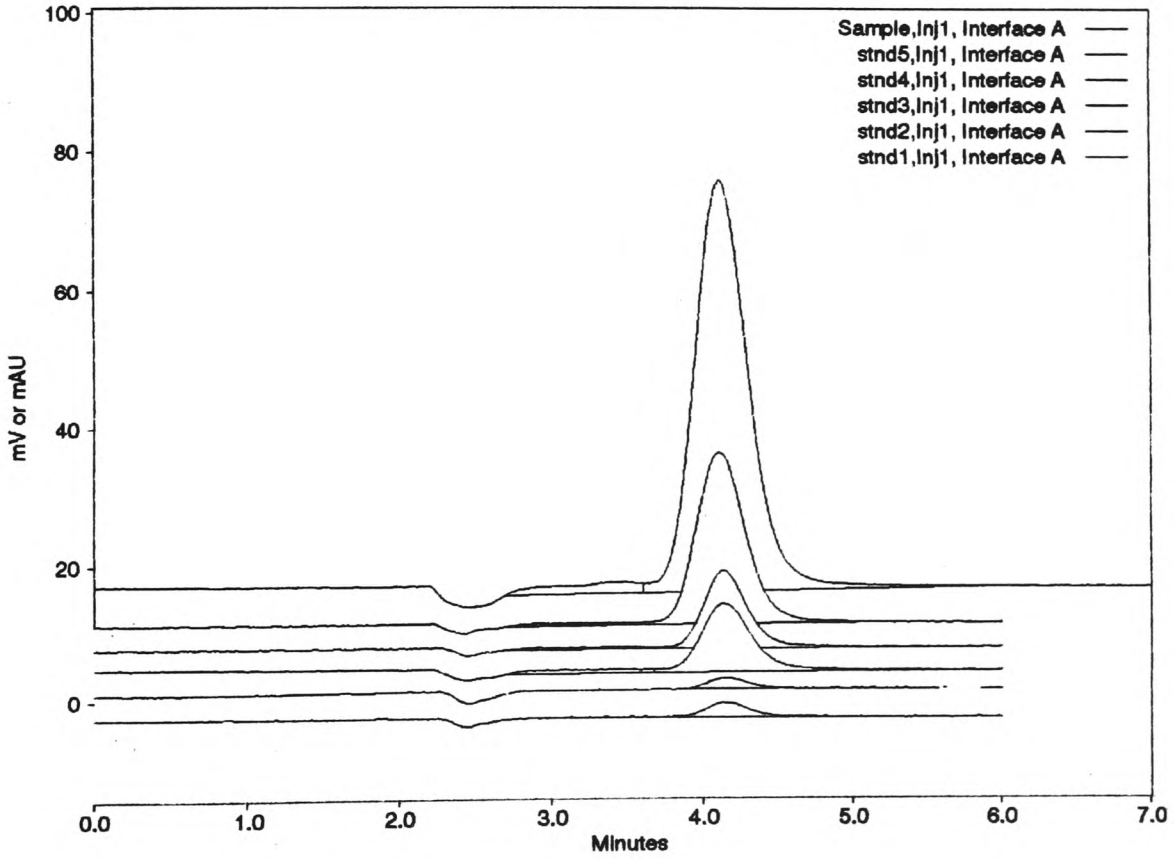
MDA in this thesis was measured using a modified method of Young and Trimble (1991). This method, which is more sensitive and specific than most spectrophotometric methods, incorporates HPLC with fluorimetric detection to measure malondialdehyde-thiobarbituric acid (MDA-TBA) adducts as illustrated in appendix 1.

MDA + TBA ⟶ MDA-TBA adduct

All chemicals were purchased from Sigma-Aldrich (Poole, Dorset), unless otherwise stated. To separate contaminating compounds with similar fluorescent properties, a Waters HPLC system was used. This included a Rheodyne 7125 injector with 50 µl sample loop, a Waters model 470 scanning fluorimetric detector and a model 745b data module (Waters-Millipore Corporation, Milford, MS, USA). The detector was pre-set at excitation 532 nm and emission at 553 nm. A 3.9 x 300mm guard column packed with µBondapak Corasil C18 was followed by a µBondapak column (Waters-Millipore, USA) to separate the TBA-MDA adduct. The thiobarbituric (TBA) reaction was carried out by mixing 250 µL of phosphoric acid (1.22 $mol.L^{-1}$), 450 µL of HPLC grade water, 50 µL of plasma or standard and 250 µL TBA (0.40* mol/L) (Sigma Ltd, Dorset, UK). To construct a calibration curve (*figure 3.5*), MDA standards (0, 0.15, 0.3, 0.6, 1.2, 2.4, 4.8 $\mu mol.L^{-1}$) were prepared using 1,1,3,3-tetramethoxypropane that yields equimolar amounts of MDA under conditions of the reaction. The reaction mixture was incubated in a boiling water bath for 1 hr in sealed glass tubes and subsequently cooled to 4^0C in ice. For chromatographic separation, the mobile phase contained 50% methanol and 50% 25mM phosphate buffer at pH 6.5, with a flow rate of 0.8 mL/min. Before column injection, samples were neutralised and de-proteinized by mixing 200 µL of plasma or standard with 360 µL HPLC grade methanol and 40 µL 1 $mol.L^{-1}$ NaOH. All chromatographs (*appendix 1 for an example of a typical chromatogram*) were printed from an adjacent dot matrix printer (Philips, E-2300, England). The intra-assay CV at an MDA concentration of 0.6 $\mu mol.L^{-1}$ was 4.9 % ($n = 9$) (*appendix 2*), while the inter-assay CV was found to be 9.1% ($n = 9$). For this, and all other assays, all samples for the same subject were analysed in the same batch.

* The TBA solution used in preparation had a concentration of 0.40 mol.L^{-1} instead of 0.44 mol.L^{-1} reported in the original paper. The lesser concentration allowed the solution to dissolve more rapidly and a saturation of TBA was not required as indicated by the higher concentration originally used. The results obtained were found to be comparable to those reported for the original assay (*Personal communication, Professor I.S Young*).

Figure 3.5 – Malondialdehyde standard peak heights



3.3.6.2 – Serum lipid hydroperoxides (LH)

Hydroperoxides in biological systems can be measured by HPLC coupled with electrochemical determination, chemiluminescence, activation of cyclooxygenase and various methods that incorporate TBA. Of these, the TBA methods are most widely used, but are also the most criticised on grounds of their ambiguity and underestimation

of extent of lipid hydroperoxides (Nourooz-Zadeh *et al*, 1994). There are currently two simple and reliable spectrophotometric methods in which lipid hydroperoxides may be determined *in vivo*. Both methods are collectively known as the 'FOX' (Ferrous Oxidation of Xylenol orange) assays, and may be differentiated by the terms FOX 1 and 2 (Wolff, 1994).

The FOX 1 assay is best suited for the determination of small levels of H_2O_2 in aqueous samples (Wolff, 1994). It is relatively free from interference by other compounds (protein, trichloroacetic acids, sodium). The FOX 2 assay is used to study H_2O_2 in liposomes and LDL cholesterol. It is best suited because of the relatively high concentrations of non-peroxidised background lipids. In this instance, alkoxyl radicals generated during the ferrous oxidation step react with native lipid, generating further hydroperoxides in a chain reaction. However, inclusion of a suitable chain breaking antioxidant such as butylated hydroxyl toluene (BHT) overcomes this problem by repairing alkyl radicals produced by the reaction of alkoxyl radicals with unsaturated lipids (Wolff, 1994).

Following the success of the original assay, Nourooz-Zadeh *et al* (1994) have modified the FOX 2 assay by using triphenylphosphine to discriminate between the background signal generated by ferric ions present in plasma and that generated by hydroperoxides in plasma.

Aqueous phase lipid hydroperoxides in this thesis were measured using a modification of the methods of Wolff (1994) and Nourooz-Zadeh *et al* (1994) (Ferrous Oxidation of Xylenol method 1: FOX 1). In dilute acids hydroperoxides oxidise ferrous ions to ferric ions, the resultant ferric ions are used as an indirect measure of hydroperoxide content, which can be detected by ferric-sensitive dyes. A blue-purple coloured complex is produced with the selective binding of xylenol orange to the ferric ions produced. The absorption can be measured at 560 nm.

$$Fe^{3+} + XO \longrightarrow \text{blue-purple complex (560 nm)}$$

Plasma samples are spiked with the enzyme catalase (Sigma, Dorset) to discriminate between authentic hydroperoxides reacting with ferrous ions and hydrogen peroxide in

the sample. All chemicals were purchased from Sigma-Aldrich (Poole, Dorset) unless otherwise stated. The method was as follows: 90 µl serum was incubated with 10 µl catalase for 30 minutes at room temperature. To this solution 900 µl FOX reagent 1 (250 µmol.L^{-1} ammonium ferrous sulphate, 100 µmol.L^{-1} xylenol orange, 100 µmol.L^{-1} sorbitol, 25 µmol.L^{-1} sulphuric acid (H_2SO_4), was added and incubated for a further 30 minutes at room temperature in the dark. Standard solutions were prepared from H_2O_2 in the range 0-5.0 µmol.L^{-1} and also incubated for 30 minutes with the FOX 1 reagent, after which the samples were centrifuged in a Beckman microfuge for 5 minutes to remove any flocculated material. The absorbance of the supernatant was read spectrophotometrically (U-2001, Hitachi, England) at 560 nm against the standard curve that was linear in the range 0-5 µmol.L^{-1}. The inter-assay and intra-assay CV was less than 4% (n = 9) and 2% (n = 9) respectively.

3.3.7 – *Determination of antioxidant concentration*

Following is a description of the procedures and techniques employed in determining various antioxidant activity in venous blood.

3.3.7.1 - Plasma ascorbic acid

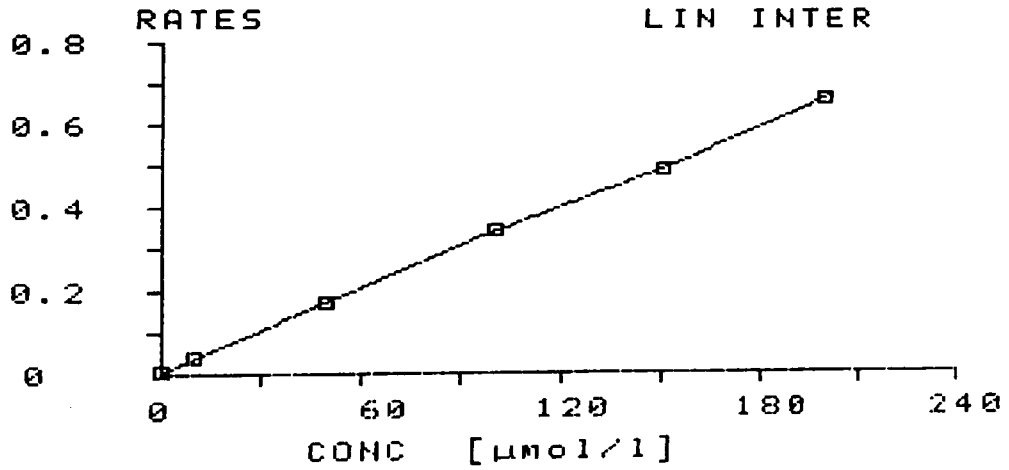
The method adopted to determine ascorbic acid concentration in plasma was that of Vuilleumier and Keck (1989). This fluorometric assay is based on the kinetics of fluorescence development by condensation of dehydroascorbic acid with 1,2-phenylenediamine. The enzymatic oxidation of ascorbic acid with ascorbate oxidase presents an assay without the need for chromatographic separation.

All chemicals were purchased from Sigma-Aldrich (Poole, Dorset) unless otherwise stated. Within 1 hr of blood collection, 100 µl of supernatant plasma was added to 900 µl of 5 % metaphosphoric acid (freshly prepared and placed on ice before use each morning of an experiment) in order to deproteinate the sample. The stabilised samples were stored at $^{-}80^{0}$C until further analysis. Upon analysis, a 2 M acetate buffer of pH 6.2 was prepared by proportioning a solution of sodium acetate trihydrate with pure acetic acid. The stock solution which consisted of 2 mg ascorbate oxidase in 1 ml of

acetate buffer was freshly prepared. The oxidising reagent was prepared by diluting 0.1 ml of the stock solution to 10 ml of buffer. A coupling reagent was fixed by adding 20 mg of 1,2-phenylenediamine to 20 ml of deionised water. A standard solution consisting of 0.5 mg of pure ascorbic acid per ml of 5% metaphosphoric acid was used in this assay (*figure 3.6*). All samples were analysed on a Roche Cobas Fara II Centrifugal Analyser at a fluorescence wavelength excitation of 340 nm.

The inter-assay CV at a concentration of 4.88 $\mu mol.L^{-1}$ ($n = 9$), 51.11 $\mu mol.L^{-1}$ ($n = 9$) and 152.85 $\mu mol.L^{-1}$ ($n = 9$) was 6.8%, 0.72% and 1.50% respectively.

Figure 3.6 – Ascorbic acid standard curve



3.3.7.2 - Plasma retinol, α-tocopherol, β-carotene, α-carotene and lycopene

The HPLC method of Catignani and Bieri (1983) and Thurnham *et al* (1988) was utilised to simultaneously determine the quantity of selected antioxidants in plasma. They were monitored by ultraviolet detection following extraction into heptane. Serum or combined working standard (retinol and α-tocopherol, retinol and α-tocopheral acetate, α and β-carotene, and lycopene) (200 µl) was pipetted into 4 ml glass tubes. α-tocopherol acetate (200 µl – Labscan) as the internal standard was added to each tube and the ethanol precipitated the protein from the serum. Heptane (Analar or 99% - 700 µl {Labscan}) containing 0.5 g/l butylated hydroxytoluene (BHT) was added to the

tubes which were then vortexed vigorously for 1 minute. The tubes were then centrifuged for 10 minutes at 3000 rpm and 500 µl of the resulting top heptane layer transferred to a set of identically labelled glass tubes. The complete contents of the standard tube contents were dried. These tubes were evaporated to dryness in a rotary evaporator (Howe, Gyrovap) under vacuum (Edwards – stage 2 pump) for 2 hrs. The samples were either assayed immediately or stoppered and stored dry in a $^{-}$70°C freezer. For HPLC analysis, the samples were reconstituted in ethanol (100 µl), vortexed and assayed using UV detection. A 45% methanol: 45% acetonitrile, 10% dichloromethane (200 ml, not filtered, degassed for exactly 5 minutes using Romil HPLC grade solvents, Analab, Dromore, N.I) mobile phase was used with a flow rate of 1.8 ml/min. The column used Hypersil ODS 5 um (Alltech), with changing wavelengths of 325 nm, 292 nm, and 450 nm to detect the retinol, tocopherol and carotenoid peaks respectively (*appendix 3*). A Thermo Separation Products Constametric 4100 pump was coupled with a Spectra-Physics AS 1000 autosampler and a Spectra-Systems SN 4000 interface and operated using PC1000 software from Thermo Separation Products.

The concentration of retinol, lycopene, α- or β-carotene and α-tocopherol in each unknown sample was calculated according to the ratio of peak height to internal standard peak height in both sample and setting standard according to the following equation (illustrated for α-tocopherol):

$$\textit{Concentration in plasma sample} = \frac{\textit{peak ht (toco.samp)}}{\textit{peak ht (toco.std)}} \times \frac{\textit{peak ht (toco.ac.std)}}{\textit{peak ht (toco.ac.samp)}} \times \textit{toco.conc.(std)}$$

toco = tocopherol, toco.ac = tocopherol acetate, samp = sample, std = standard, conc = concentration, ht = height.

A quality control serum sample was assayed at the start of every batch of samples. The inter-assay CV for α-tocopherol was 5.4% (n = 7) and 10% (n = 7) for β-carotene. The CVs for retinol, lycopene and α-carotene were similar to those for β-carotene. The addition of internal standard allowed the calculation of an intra-assay CV of 4.9% (n = 10).

3.3.8 – Muscle damage markers

3.3.8.1 – Total phosphocreatine kinase activity

Creatine kinase (CK) concentration was measured using the Kodac Ektachem clinical chemistry slide. The slide is a dry, multilayered self-containing analytical element coated on a transparent polyester support. 11 μl of specimen is placed on the slide and evenly distributed by the spreading layer. The layer also contains N-acetylcysteine (NAC) to activate CK without pre-treating the sample. The reagent layer contains an adenylate kinase inhibitor, diadenosine pentaphosphate, as well as the enzymes, buffers and dye precursors necessary for the measurement of CK activity. When the sample is deposited on the slide, CK catalyses the conversion between creatine phosphate and ADP to form creatine and ATP, as shown in the following reaction sequence:

$$\textbf{Creatine Phosphate + ADP} \xrightarrow{\textit{Creatine kinase}} \textbf{Creatine + ATP}$$

In the presence of glycerol kinase, ATP phosphorylates glycerol to L-α-glycerophosphate. Oxidation of L-α-glycerophosphate to dihydroxyacetone phosphate and H_2O_2 occurs in the presence of L-α-glycerophosphate oxidase. Finally, a leuco dye precursor is oxidised by H_2O_2 in the presence of peroxidase to form a dye. Reflection densities are monitored during incubation. The rate of change in reflection density is then converted to the measurement of enzyme activity. The inter assay CV was 2.5%.

3.3.8.2 – Serum myoglobin

Myoglobin concentration was analysed using the Chiron Diagnostics ACS:180 Automated Chemiluminescence System. The system uses constant amounts of two different antibodies. The first antibody, a polyclonal goat anti-myoglobin antibody is labelled with acridinium ester. The second antibody, in the solid phase, is a monoclonal mouse anti-myoglobin antibody covalently coupled to paramagnetic particles.

Serum myoglobin concentration was assessed automatically using the following procedure:

1. 10 µl of serum was dispensed into a curvette.
2. 100 µl of lite reagent (1) was added and incubated for 2.5 mins at 37°C.
3. 200 µl of solid phase reagent (2) was added and incubated for a further 5 mins at 37°C.
4. The curvette was subsequently separated, aspirated and washed with regent water.
5. 300 µl of reagent 1 and 2 was then added to initiate the chemiluminescent reaction.

Serum myoglobin is determined by the direct relationship of the concentration present in the sample and the volume of relative light units (RLUs) detected by the analyser. The inter-assay CV was 3.5%.

3.3.9 – Whole blood neutrophil and leukocyte analysis

Total and differential leukocyte analysis was determined using a COULTER® GEN·S™ automated haematology analyser (Coulter Corporation, Miami, U.S.A). The Coulter method counts the total white blood cells (WBC) by detecting and measuring changes in electrical resistance when a cell in a conductive liquid goes through a small aperture. Each cell suspended in conductive liquid acts as an insulator. As each cell goes through the aperture, it momentarily increases the resistance of the electrical path between two submerged electrodes, one located on each side of the aperture. This causes an electrical pulse that can be counted.

The WBC count is the number of leukocytes measured directly multiplied by the calibration factor. This number (n) may be expressed as:

$$WBC = 10^3 \text{ cells}/\mu l$$

The WBC differential analysis and classification (*e.g.* neutrophils), occurs in the flow cell where (1) low-frequency current measures volume, (2) light bounces off the individual WBC cells which characterises cellular surface, shape and reflectively.

The absolute number of neutrophils are derived from the WBC count and differential % as shown below:

$$\text{NE } (10^3 \text{ cells}/\mu\text{l}) = \frac{\text{NE\% x WBC count}}{100}$$

3.3.10 – Magnesium analysis

Plasma magnesium (Mg) concentration was measured on a Ortho Vitros 950 analyser using the Vitros clinical chemistry slide (Ortho-Clinical Diagnostics, Rochester, U.S.A). The slide is a dry, multilayered, analytical element coated on a transparent polyester support. 10 µl of specimen is placed on the slide and evenly distributed by the spreading layer. The layer also contains a calcium chelator and a dye necessary for the measurement of Mg activity as shown in the reaction sequence below. Mg (both free and protein-bound) from the sample reacts with the formazan dye derivative causing the high Mg affinity of the dye to dissociate Mg from binding proteins. The resulting Mg-dye complex causes a shift in the dye absorption maximum from 540 nm to 630 nm. The amount of dye complex formed is proportional to the Mg concentration present in the sample and is measured colorimetrically by reflection density.

$$Mg^{+2} + Ca^{+2} \xrightarrow{\textit{chelator}} Mg^{+2} + Ca^{+2}\text{-chelator complex}$$

$$Mg^{+2} + \text{formazen dye derivative} \longrightarrow Mg^{+2}\text{-dye complex}$$

3.3.11 - Blood glucose analysis

Blood glucose was measured by dry chemistry slide technology on an Ortho Vitros 950 analyser. 10 µl of whole blood is placed on the slide where the spreading layer promotes the uniform distribution of the sample and allows an even penetration of solute molecules into the underlying reagent layer. The oxidation of blood glucose is catalysed by glucose oxidase to generate hydrogen peroxide and gluconate. Oxidative coupling caused by peroxidase in the presence of dye precursors produces a dye, as shown in the following reaction sequence:

$$\beta\text{-D-glucose} + O_2 + H_2O \xrightarrow{\text{glucose oxidase}} \text{D-gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminoantipyrine} + \text{1,7-dihydrooxynaphthalene} \xrightarrow{\text{peroxidase}} \text{red dye}$$

The intensity of the dye is measured colorimetrically at a wavelength of 540 nm. The inter-assay CV at a glucose concentration of 6.1 mmol.L^{-1} was 2.05% and at 14.7 mmol.L^{-1} was 1.12%.

3.3.12 – Blood glycosylated haemoglobin (HbA_{1c}) analysis

The HPLC method of Philcox *et al* (1992) was utilised to determine glycosylated haemoglobin (HbA_{1c}) percentage. 50 mL of EDTA-treated blood was mixed with 10 mL of physiological saline. The sample was segmented by centrifugation for 5 mins at 500 rpm, and the supernatant fluid discarded. 2 mL of 5 mmol.L^{-1} KCN acidified to pH 4.7 with acetic acid was added to the cell pellet and mixed thoroughly to ensure haemolysis. Cellular debris was discarded after centrifugation at 4000 rpm for 5 mins. 10 µl of sample was subsequently analysed on a pre-prepared HPLC system from Waters (Milford, MA) using a Mono S HR 5/5 Cation-Exchange column (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

3.3.13 – Whole blood lactate ($[La^-]_B$) analysis

Approximately 50 µl of arterialised capillary blood was collected in a capillary tube (Analox Instruments Ltd) lined with heparin, fluoride and nitrite (*glycolytic inhibitors*) and mixed for 4 minutes. The appropriate volume of blood required for analysis was achieved with the aid of a micropipette (Gilson Microman), which was previously calibrated to 7 µl. The concentration of $[La^-]_B$ was measured using an automated electrochemical analyser (Analox PGM7 Champion, London, UK). Fresh buffer was added to L-lactate:oxygen oxidoreductase (LOD) at an ambient temperature of 21°C and placed inside the analyser. Injection of the sample into the cuvette activates an oxidation-reduction reaction catalysed by LOD at a pH of 6.5. The maximum rate of oxygen consumption during the reaction is directly related to the concentration of

lactate in the sample. The analyser was calibrated prior to blood sampling using 7 μl of a known calibration standard (8 $mmol \cdot L^{-1}$ of bovine $[La^-]_B$). 7 μl of a quality control sample (Analox lactate/pyruvate quality control serum) was used to ensure reagent activity and to correct calibration. Duplicate samples were analysed and the mean of the two was taken as the definitive value.

This method of $[La^-]_B$ analysis has been validated against a spectrophometric method (Sigma-Aldrich, Poole, Dorset).

3.4 - Anthropometric measurements

Each subject was instructed to wear shorts and remove footwear prior to the assessment of body mass index. Height was measured to the nearest 0.1cm and weight to the nearest 0.1kg using a free-standing stadiometer (Seca, Cardiokinetics, Salford, UK). The accuracy of the stadiometer was checked by calibrating the scales with a 1 kg free weight (*weight*) and a tape measure (*height*).

3.4.1 – Body fat

Harpenden skinfold callipiers (John Bull, British Indicators Ltd, Berkshire, UK) with a constant spring pressure of $10g/mm^2$ were accurately calibrated with a Vernier scale and used to measure skinfold thickness at the biceps, triceps, subscapular and suprailliac sites on the left hand side of the subjects body. Three repeated measurements were obtained from each skinfold site. Percentage body fat was estimated from the sum of four skinfolds using the procedures of Durnin and Womersley, (1974). The same investigator performed all measurements.

The CV of repeated skinfold measurements has been estimated at 5%, and has been demonstrated to correlate highly ($r = 0.83$ to 0.89; $P < 0.05$) with hydrostatic weighing (Durnin and Womersley, 1974).

3.5 - Cardiovascular measurements

3.5.1 - Laboratory heart rate

Two methods of determining heart rate were utilised. One method involved using an electrocardiograph (ECG) calibrated short-range telemetry device (Polar Electro Oy, Finland). Two electrodes attached to an adjustable belt that comprised a transmitting device were placed on either side of the sternum. A watch that received magnetic waveforms was fixed to the handlebars of the cycle ergometer (Monark 814, Varberg, Sweden), and this allowed continuous wireless reception and internal recording of heart rate expressed in beats per minute ($b.min^{-1}$).

The other method involved using a 3 lead ECG system (Life pulse, HME, England). The skin surface was prepared using a scouring pad, cotton wool and clinical swabs. To ensure accurate readings by minimising resistance across the electrodes, body hair was carefully removed using a razor (Wilkinson, UK). Three ECG electrodes (Ag/AgCL, Skintact, Austria) were secured to the following anatomical sites: right chest (below right clavicle, midway between sternum and shoulder), left chest (below left clavicle, midway between sternum and shoulder), upper abdomen region (upper left side of abdomen). The decision to place the electrons on these sites was made on the basis of limiting the possibility of artefactual readings recorded during exercise. Transpore surgical tape (3M, USA) was used to secure the electrodes and trailing wires in place. The heart rate monitor screen was positioned out of subject sight.

3.5.2 - Arterial oxygen saturation (SaO_2)

Arterial oxygen saturation (SaO_2) was measured using a finger pulse oximeter (Nonin, 8800, cardiorespiratory oximeter, Plymouth, U.S.A). Care was taken to position the sensor on the top and bottom of the end of an index finger. The light emitter portion was placed on the finger nail side and the detector on the side opposite to the nail. The pulse oximeter shines red and infrared light through the tissue and detects the fluctuating signals caused by arterial blood pulses. The ratio of fluctuation of the two colour signals received determines the SaO_2:

$$SaO_2 = f \quad \frac{\text{In (min/max)Red}}{\text{In (min/max)IR}}$$

f = function

Red/IR = Red light measured at 660 nm/infrared light at 910nm

The accuracy of this measurement has been estimated at $\pm$ 2%.

3.5.3 - Blood pressure

Systemic arterial blood pressure (BP-mmHg) was measured in the brachial artery using a mercury sphygmomanometer (Accoson, Cardiokinetics, UK) and stethoscope (Littmann, 3M, USA). Systolic pressure was noted when clear muffling of repetitive tapping sounds (Korotkoff) became apparent and diastolic pressure was noted when the repetitive sounds (Korotkoff) diminished (Tortora and Grabowski, 1996).

3.6 - Respiratory measurements

3.6.1 - Medgraphics CPX/D

On-line oxygen uptake/consumption was measured using a Medgraphics gas analysis system (Medgraphics, CPX/D, Manchester, UK), which generates real-time, breath by breath oxygen uptake. Subjects breathed through a previously sterilised rubber mouthpiece attached to a 26g (0.9oz) preVent pneumotach (valve dead space = 20ml) and transducer. The pneumotachograph was calibrated at 5 different flow rates using a 3 L calibration syringe (Medgraphics, U.S.A) to verify a linear response prior to exercise. Gas signals were directed to a waveform analyser which subsequently transforms the analog signals to correlate with flow. The volume was calculated by a computerised integration of flow relative to time. Dried expired gas samples were directed to fast responding infra red CO_2 and Zirconium O_2 analysers, which were previously calibrated using known gas concentrations:

Reference gas: 21% O_2, balanced nitrogen (N_2)

Calibration gas: 5% CO_2, 12% O_2, balanced N_2.

(Medgraphics, U.S.A)

Respiratory parameters expressed at BTPS were sampled every 30's and printed on-line (Citizen, Swift 200, UK).

3.6.2 - Douglas bag method

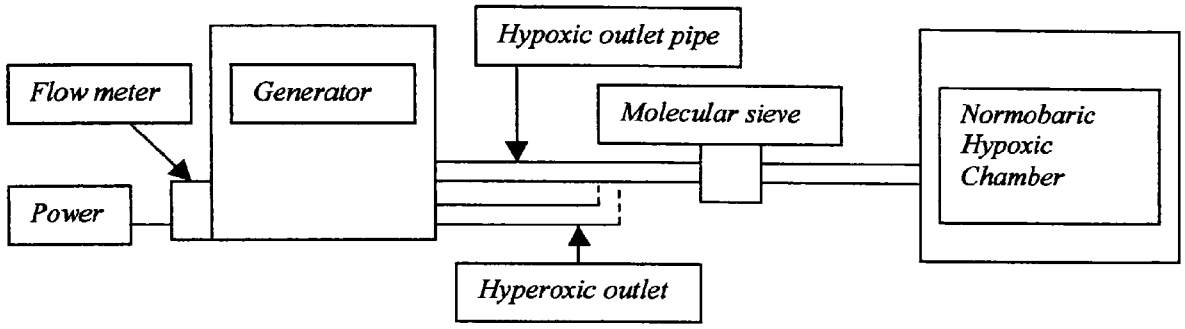
Off-line gas analysis was determined using the '*gold standard*', Douglas Bag method. Volunteers breathed through a large 2–way non-returnable breathing valve (Hans-Rudolph, Kansas, U.S.A). Expired air was directed into a Douglas Bag (150L), through plastic tubing and a two-way stopcock valve (Hans-Rudolph, Kansas, U.S.A). The collection of expired gas was hand timed using chronography (Timex, UK) to the nearest whole breath for a specific time period (*i.e.* 60 secs). Samples of expired air were dried using 97% anhydrous calcium sulfate ($CaSo_4$) crystals (Drierite, U.S.A) and presented to a paramagnetic O_2 and infra-red CO_2 analyser (Servomex 1400B4, Sussex, UK) for the determination of percentage O_2 and CO_2. The repeatability of the O_2/CO_2 analysers has been documented as $\pm$ 0.1%. Before use, the analysers had been calibrated using specialised gas mixtures ([N_2-O_2 free], [5% CO_2, 17% O_2, balanced N_2]) (Spantech, Surrey, UK). The volume of expired gas was measured using a dry gas meter (Harvard Ltd, Edenbridge, UK). Computation of oxygen uptake ($\dot{V}O_2$) and carbon dioxide production ($\dot{V}CO_2$) corrected to STPD was computed using the Haldane transformation equation (Wasserman *et al*, 1994).

3.7 – Hypoxic chamber

The normobaric hypoxic and normoxic chamber (width 1.47m, length 2.68, height 2.44m, capacity 9612 L) (Hypoxico training systems, California, U.S.A) consisted of a molecular sieve which determined the percentage of inspired fraction of oxygen (F_IO_2). Ambient O_2 (20.93%) was drawn into a generator connected to a flow meter. An hypoxic outlet pipe passed through a molecular sieve into the chamber for the hypoxic exercise trial (16% F_IO_2), whilst the hypoxic and an adjacent hyperoxic outlet pipe where joined to regulate the concentration of F_IO_2 for the normoxic exercise trial (20.93% F_IO_2) (*figure 3.7*). A paramagnetic O_2 analyser as previously described was placed inside the chamber, blind to the exercising subjects, to monitor atmospheric O_2

concentration. All pipes leading into the chamber were identical in sound and disguised for all trials. Five generators in total were used (*appendix 4 for pilot work*).

Figure – 3.7 – Schematic overview of hypoxic chamber



3.8 - Cycle ergometer

The same cycle ergometer (Monark 814, Varberg, Sweden) was used for all experiments. A complete calibration was performed prior to each experiment following the guidelines of Coleman (1996). The underlying principle involves the application of kinetic energy to the flywheel. The flywheel is braked by means of a brake belt located on the flywheel. Workload was adjusted by manually placing weights on the attached basket. A battery controlled electronic output monitor displayed pedalling velocity (revs.min^{-1}). The ergometer had an adjustable seat and handlebars, which were set to suit each individual subject. Toe-clips attached to the pedals of the ergometer secured the subject's feet to the pedals.

3.9 - Rate of perceived exertion

Rate of perceived exertion (RPE) was estimated using the Borg's Scale (1973). A numeric value, which ranges from 6-20 may be selected to indicate a perceived rate of exertion from "*very very light*" to "*maximum*".

3.10 – Temperature and humidity regulation

Room temperature was measured using a wall-mounted temperature and humidity gauge (Thermo-Hydro), which measured temperature and humidity to the nearest 0.1°C and 0.1% respectively. Barometric pressure was obtained from a wall-mounted barometer.

3.11 - Dietary analysis

For 3 days preceding the experimental phase, each volunteer recorded the quantity and type of food and beverage consumed. This data was analysed using a commercial software package (Nutri-check, Health Options Limited, Nottingham, UK) (*study one*), and a computerised software package (Balanced Diet - version 1.12) (*pilot study and studies two and three*). Volunteers were instructed to maintain their usual dietary pattern.

3.12 - Statistical analysis employed

Computerised statistical analysis was performed using the SPSS social statistics package - version 9.0 (Surrey, UK).

3.12.1 – Power of the test

A preliminary attempt was made to assess the sample size required to detect an intervention effect at $P < 0.05$, however it must be emphasised that power calculations for complex MANOVA determination are inherently difficult. The employed method of Altman (1980) was used and incorporated the critical difference for the main biochemical parameters assessed (Fraser and Fogarty 1989, Bailey 1997) (*see Chapter 4*). Where appropriate retrospective power calculations are included.

3.12.2 – Descriptive statistics

Data were analysed using parametric statistics following mathematical confirmation of a normal distribution by repeated Kolmogorov-Smirnov tests. The alpha was established at $P < 0.05$ and all descriptive values are reported as means $\pm$ 1SD.

3.12.3 – Comparative statistics

Independent sample *t*-tests were used to compare variable differences between groups.

Study 1 and 2: Resting and post-exercise data were analysed using a two-way [A x (B)] mixed analysis of variance (ANOVA) which incorporated one between [study 1, *group*: *normoxic vs. hypoxic* (*absolute*) *vs. hypoxic* (*relative*)] (study 2, group: *diabetic vs. control*) and one within (time: *rest vs. exercise*) subjects factor.

Study 3: Resting and post-exercise data were analysed using a three-way [A x B (x C)] mixed analysis of variance (ANOVA) which incorporated two between (groups: *diabetic vs. control and ascorbic acid vs. placebo*) and one within (time*:* *rest vs. exercise*) subjects factor. A two-way [A X (B)] mixed ANOVA was used to determine group differences listed in tables 7.0 and 7.2.

Following a significant interaction effect (*time x group or time x group x treatment*), within subjects factors were analysed using bonferroni-corrected paired samples *t*-tests. Between subject differences were analysed using a one-way ANOVA with *a posteriori* Tukey Honestly Significant Difference (HSD) test.

The linear relationship between two dependent variables was established using Pearsons Product Moment correlations. The Δ change (*rest minus exercise, and pre-supplementation minus pre-exercise*) was used particularly when performing correlations between two dependent metabolites.

3.13 – Preliminary quality control experiments

3.13.1 - INTRODUCTION

In medicine and biology, free radicals have received much attention of late, primarily due to the fact that they appear to be implicated in various aspects of metabolism (Buettner, 1987). Electron Spin Resonance (ESR) spectroscopy is the least ambiguous method for free radical detection and identification (Thornalley 1986, Buettner 1987); however, its use in exercise physiology *per se* is a relatively new phenomenon. With this in mind a number of quality control experiments were completed in order to complement the validity of ESR data reported in this thesis.

3.13.2 – METHODOLOGY

The following experiments were primarily designed to detect the presence of artefactual free radical species, and comprised the following conditions:

(a) - Empty Bruker cavity
(b) - Blank ESR tube
(c) - Degassed toluene
(d) – Untreated whole blood
(e) – Serum with no PBN
(f) - Degassed toluene and PBN
(g) – Presence of PBN oxidation

Materials

Materials included a precision bore glass ESR tube, HPLC grade toluene, physiological saline, and the nitroxide spin-trap, PBN. The degassing and treatment of all materials are the same as outlined in section 3.2.7.

PBN was chosen as the spin trap of choice in this thesis for a number of imperative reasons:

(1) PBN has been validated as a spin trap capable of identifying lipid-derived free radicals generated in type 2 diabetes (Anderson *et al,* 2001) and in exercise (Ashton *et al*, 1998).

(2) PBN is not over sensitive to light, oxygen or water.

(3) In inert solvents, such as benzene or toluene, any photolytic degradation that may occur does not produce significant concentrations of nitroxide radicals.

(4) It is relatively hydrophilic.

(5) Commercially available PBN is normally of sufficient purity to exclude the need for further purification.

(6) PBN spin traps and their adduct are known to be relatively stable and long-lived.

(7) Relatively inexpensive compound.

(Finkelstein *et al* 1980, Janzen 1980).

There are a few disadvantages however, associated with the use of PBN that are not evident with the use of other spin trap molecules (*e.g.* 5,5 dimethyl-1-pyroline-N-oxide [DMPO]).

(1) Slower trapping rate constant.

(2) Spin adduct spectra less informative.

(Janzen, 1980)

ESR operating conditions

All analysis was completed at room temperature on a Bruker EMX series X-band ESR spectrometer (Bruker, USA, software version 3.2) fitted with a Bruker TM_{110} cavity using the same operating conditions as outlined in section 3.2.7. Spectrometer and spectra reliability was tested using a stable synthetic radical (diphenylpicrylhydrazil (DPPH) – no: -9702D187).

Blood sampling

All blood sampling and handling followed the procedures outlined in sections 3.3.1 and 3.3.2.

3.13.3 – RESULTS

No paramagnetic free radical species were detected in conditions *a*, *b*, *c*, *d*, or *e* (*Spectra shown in appendix 5, 6, 7, 8 and 9*). However, a triplet of doublets was observed in condition *f*, as shown in figure 3.9. A typical spectra of PBN oxidation is shown in figure 3.10.

An additional experiment was needed to determine the stability of the PBN adduct over a 2.5 hr duration at room temperature. Results indicate that PBN adduct stability decreases linearly over time as shown in figure 3.8. However, when the PBN adduct was flash frozen in liquid nitrogen and subsequently measured 2.5 hrs later, the rate of decline was minimal.

Figure 3.8 – The relationship between venous PBN adduct decay and time

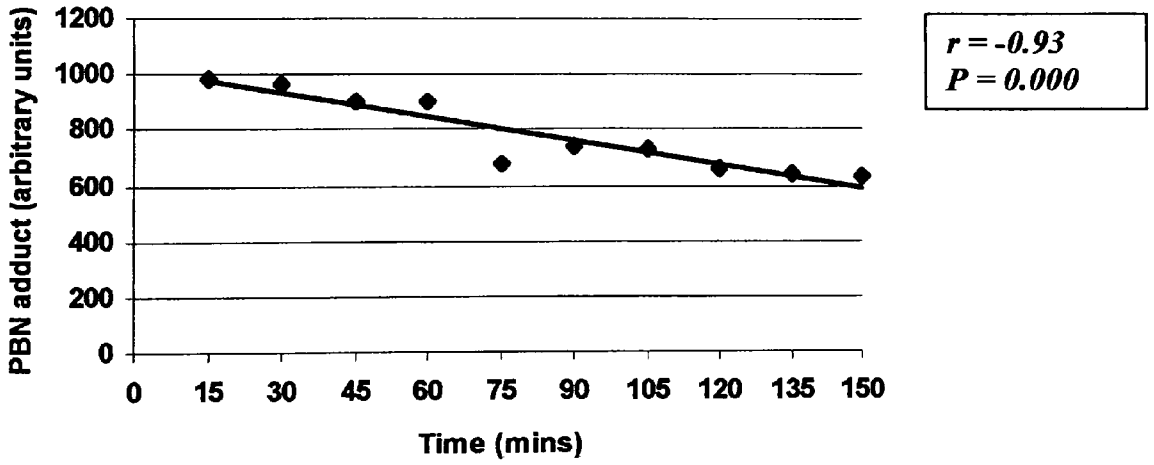


Figure 3.9 – Typical ESR spectra of degassed toluene and PBN (in vitro sample)

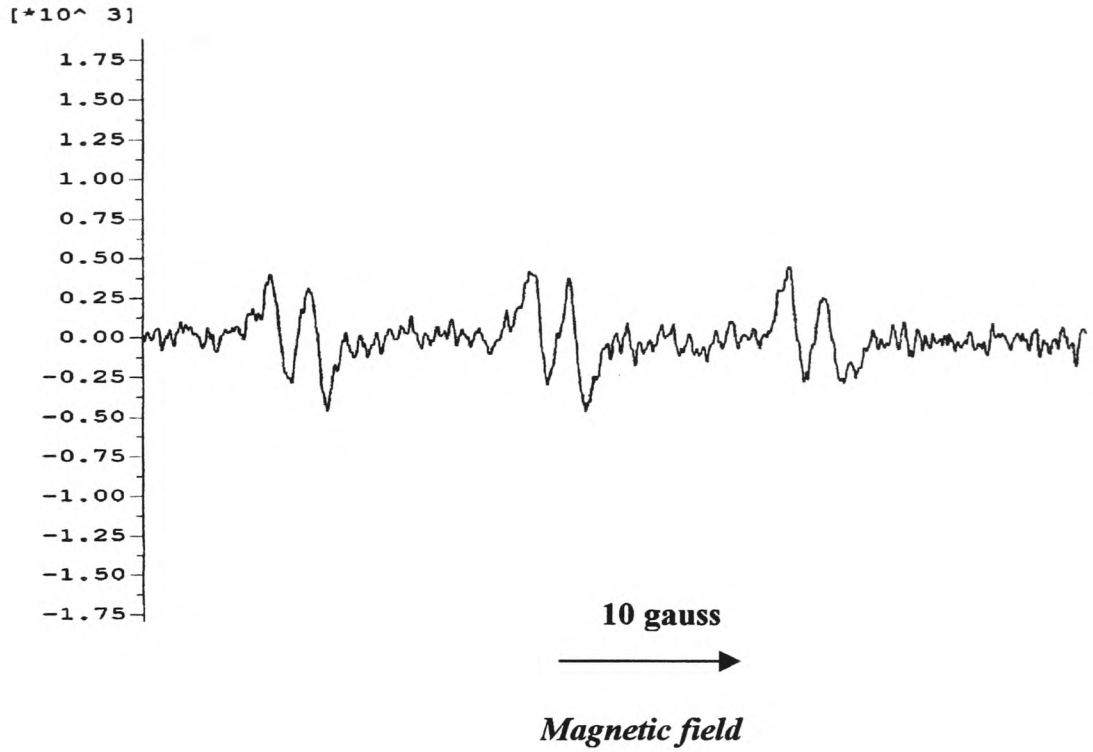
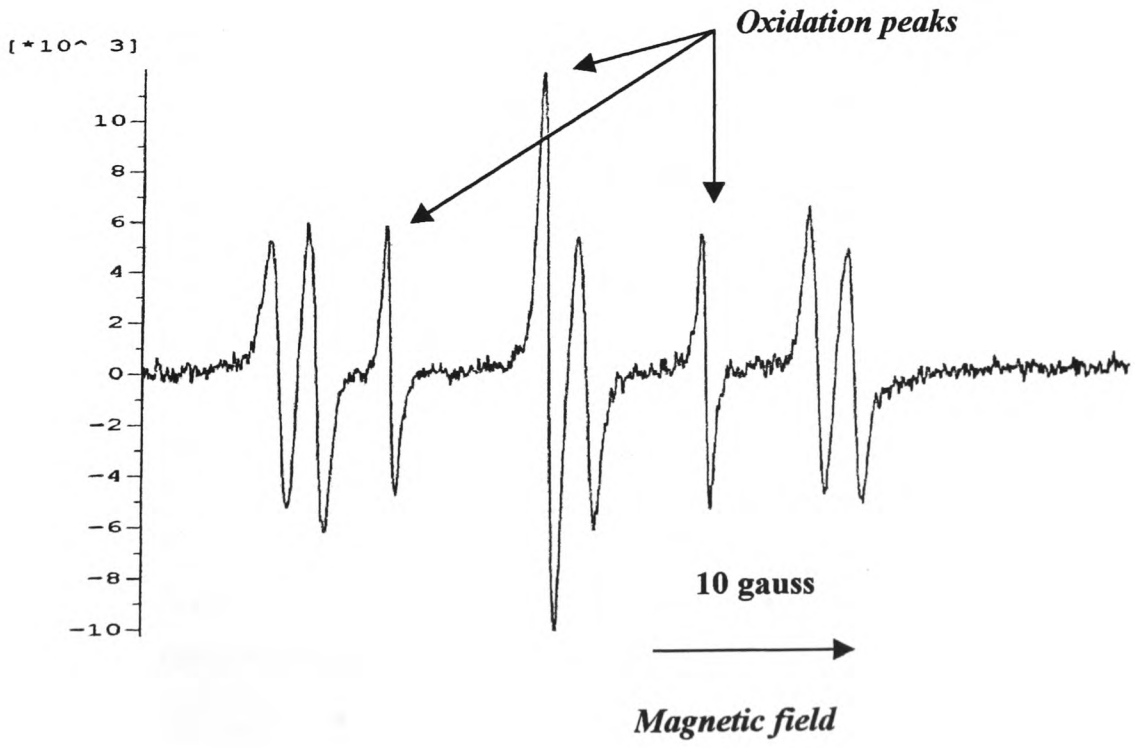


Figure 3.10 – Typical spectra of PBN oxidation (ex vivo biological sample)



3.13.4 – DISCUSSION

The spectra shown in figure 3.9 clearly illustrate a "*classical*" triplet of doublets, characteristic of a PBN nitroxide adduct. The hyperfine coupling (splitting) constants were measured at a_N 14.2 gauss and $a\beta_H$ 2.1 gauss, which would tentatively identify the radical species as being either oxygen or carbon-centred (Thornalley, 1986). More specifically, these coupling constants closely resemble those of both the PBN trapped superoxide anion (a_N 14.3 gauss and $a\beta_H$ 2.3 gauss) and hydroxyl radical (a_N 14.1 gauss and $a\beta_H$ 2.0 gauss) (Thornalley 1986, Buettner 1987). The origin of the species is poorly understood and warrants further investigation. However, a possible source under the circumstances may include ultraviolet light (Buettner, 1987). Furthermore, it is known that nitroxide radical adducts may be generated or transformed by the passing of aqueous solutions through steel needles and plastic based solids (*e.g.* pipette tips), thus care in sample preparation and handling is essential (Davies and Timmins, 1996). Due to the low concentration of free radicals detected (0.74 arbitrary units) from degassed toluene and PBN, it is not necessary to correct/adjust the biological PBN adduct in future work, as all samples are treated in the same way.

The spectra shown in figure 3.10 also demonstrate a triplet of doublets with hyperfine coupling constants of a_N 13.6 gauss and $a\beta_H$ 1.9 gauss. The free radical species may also be identified as being either oxygen or carbon-centred, yet in contrast to the above species mentioned, are lipid derived alkoxyl or peroxyl radicals, possibly derived from oxidative damage to phospholipid cellular membranes. The coupling constants observed in this spectra are identical (a_N 13.6 gauss and $a\beta_H$ 1.9 gauss) to those of patients undergoing elective cardioplegia (Tortolani *et al*, 1993). Moreover, these authors also attribute the detection of alkoxyl radical formation to membrane lipid breakdown. However, the clarity of the spectra is overshadowed by the presence of clear oxidation of the PBN molecule. PBN oxidation (PBNOX) is usually characterised by three single peaks separated by an equal distance and a strong smell of '*bitter almonds*' from the PBN batch (*Personal communication, Dr G Timmins*). It occurs when there is a carbonyl group at the β-position of the PBN molecule, and when the hydrogen atom adducts to the trap (*i.e.* PBN-H) (Davies and Timmins, 1996). The latter

can also result from spin trap reduction and subsequent protonation (Davies and Timmins, 1996).

3.13.5 – CONCLUSION

PBN is generally a very stable nitrone spin trap (Janzen, 1980), however data collected during the quality control studies demonstrate that artefactual free radicals can be observed and that PBN can undergo oxidation. Nevertheless, these data show that PBN is an appropriate spin trap since other nitrones can require further purification and are more susceptible to artefactual radical production and oxidation (*e.g.* DMPO).

Chapter 4

Pilot Studies

The Determination of (1) Biological Variation and (2) Critical Difference

4.0 – INTRODUCTION

Evidence suggesting that free radicals increase susceptibility to certain disease processes, has stimulated much research in the area of oxidative stress (Halliwell 1994, Ames 1989, Diplock 1994). Likewise, exercise physiologists have primarily adopted the markers of lipid peroxidation to determine whether or not exercise causes damage to membranes (Alessio *et al* 2000, Viinikka *et al* 1984). These studies commonly aim to describe the metabolic and biochemical status of certain groups and to perhaps examine the effect of interventions. In many cases the metabolic status of an individual may be represented by measurements obtained at a specific time of day, however, some parameters have circadian rhythms that may be daily, monthly, or seasonal. In addition, studies often pay little attention to how such measurements fluctuate from person to person, with most individuals having inherent fluctuations, which can be described as random variation close to a homeostatic set point (Fraser and Fogarty, 1989). Fraser and Fogarty (1989) refer to this set point as the within subject biological variation. Within subject biological variation has several sources, incorporating such factors as assay variation, variation in technique (*e.g.* blood sampling), day-to-day variation in measurement conditions, and underlying *biological* variation, which according to Widjaja *et al* (1999) "*reflects a multitude of exogenous and endogenous perturbations on biological systems*".

In addition to the quantification of biological variation, and of equal importance, is the determination of critical difference. Critical difference according to Fraser and Fogarty (1989) is the change that must occur before a true physiological difference can be claimed. For example, whilst the response to physical exercise may change the concentration of a metabolite significantly ($P < 0.05$), the magnitude of that change may be physiologically insignificant. Therefore, a clear distinction between statistical and physiological significance must be determined before drawing conclusions as to whether any *real* physiological or biochemical changes have occurred.

The concept and importance of biological variation and critical difference is well recognised in clinical biochemistry, but is often neglected in the field of exercise physiology. Reference data exist for a wide range of clinical and haematological

analytes, however, there are currently no reference data for any oxidative stress parameter in which the exercise physiologist may refer to.

With this in mind, the present pilot studies were designed to establish the within subject biological variation and critical difference values of the oxidative stress and antioxidant parameters used in this thesis.

4.1 - METHODOLOGY

Subject characteristics

Ten (n = 10) apparently healthy male volunteers were recruited from the student population of the University of Glamorgan to participate in the present study (*table 4.0 for subject characteristics*). This cross-sectional approach was adopted in order to maximise the variation of subject data. Ten subjects were chosen as it has previously been suggested that valid estimates of the components of variation can be obtained from a relatively small group of subjects over a reasonably short period of time (Fraser and Harris, 1989). Subjects had no known physician diagnosed diseases or ailments assessed by a medical history questionnaire. After the nature and risks of the experimental design were explained to the subjects, a questions and answering session originated to discuss any detail before written informed consent was given by the subjects in the presence of an independent witness. All subjects were fully informed that they were free to withdraw from participation at any time.

Table 4.0 – Age and physiological characteristics of subjects

Dependent variable	***Mean ± SD***
Age (yrs)	24 ± 3
Stature (cm)	178 ± 7.1
Body mass (kg)	80 ± 14
Body mass index (kg/m^2)	25.1 ± 3
Bodyfat (%)	14 ± 6

Experimental design

The local Medical Research Ethics Committee (Bro Taf) granted approval for this study. Subjects were instructed to abstain from any dietary antioxidant intervention for six weeks prior to the experimental protocol. Subjects were also instructed to refrain from exercise, caffeine and alcohol for 48 h before the experimental period began and to maintain their usual dietary pattern. Dietary composition and caloric intake in the 72 h before the exercise test was recorded by means of a food diary and assessed using a standard nutritional assessment package as described in methodology section 3.11. Each subject was randomly assigned to an individual slot within a two-week period.

Anthropometric measures

Subject body mass and stature was determined as outlined in methodology section 3.4.

Body fat assessment

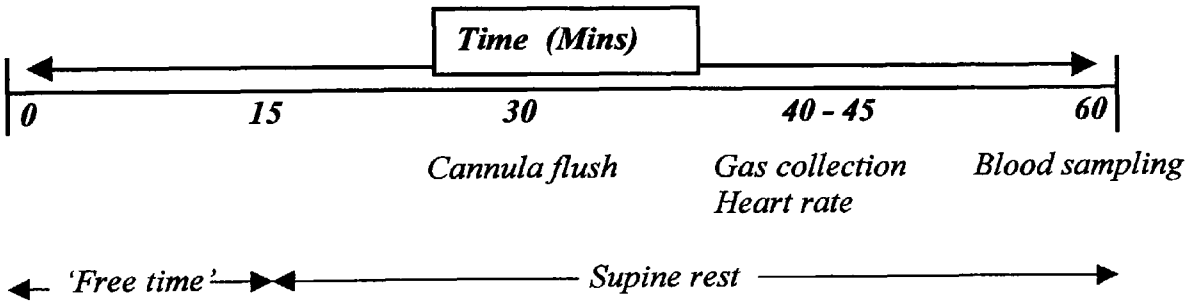
Body fat was determined using skin fold callipers and calculated according to the method of Durnin and Womersley (1974). A detailed description can be obtained by referring to methodology section 3.4.1.

Experimental protocol

Subjects arrived at the laboratory at 8.00am after a 12 hr overnight fast. The subjects arm was placed into a heated water bath to maximise blood flow before an intravenous cannula was placed into a prominent forearm vein (refer to methodology sections 3.3.1 and 3.3.2.). Whilst in the supine position, blood was drawn from each individual by the same phlebotomist (this practice helped minimised pre-analytical variance) once every hour for an eight hour period (*i.e.* 9.00am to 5.00pm). Immediately after blood sampling, subjects were allowed to slowly stand up and walk for 15 mins if desired, confined to the laboratory. The cannula line was '*flushed*' with 3ml physiological saline every 30 mins to keep the line patent (*figure 4.0*). Subjects abstained from standing during the 45 mins prior to each blood collection. Arterialised capillary blood was taken at the same time as venous collection for packed cell volume and

haemoglobin determination (methodology sections 3.3.4 and 3.3.5 for respective details). Subjects abstained from food throughout the day; however, controlled water intake (2 ml·kg body weight) was permitted after each blood sample given. Resting oxygen uptake ($\dot{V}O_2$) was monitored periodically throughout the day (*figure 4.0*) using an on-line automated gas analysis system as described in methodology section 3.6.1. Resting heart rate using a short-range telemetry device was also measured at periodic times throughout the day (*figure 4.0*). A detailed description of procedures may be found in methodology section 3.5.1. The laboratory was regulated for temperature (20.9°C) and humidity (54%) as described in methodology section 3.10.

Figure 4.0 – Schematic representation of collection procedures within a 1 hr period



Haematological analysis

To minimise analytical variance, all samples from each individual were assayed in the same batch. Samples from all individuals were assayed with the same batch of reagents, standards, and quality control materials, and the same two individuals performed all analysis. All venous blood samples were treated as reported in methodology sections 3.3.1 and 3.3.2.

PBN adduct preparation and analysis

Fresh PBN was prepared each morning of experimental day. The PBN adduct was analysed and the spectra measured on a Bruker EMX X-band ESR spectrometer. A detailed description of PBN preparation and adduct analysis can be obtained by referring to methodology section 3.2.7.

LIPID PEROXIDATION MEASURES

Malondialdehyde (MDA) analysis

MDA was measured by HPLC in EDTA plasma using a modified method of Young and Trimble (1991). A detailed description can be obtained by referring to methodology section 3.3.6.1.

Lipid hydroperoxide (LH) analysis

LH was measured spectrophotometrically in serum using a modified method of Wolff (1994) and Nourooz-Zaheh *et al* (1994) (***F***errous ***O***xidation of ***X***ylenol orange method; FOX 1). A detailed description can be obtained by referring to methodology section 3.3.6.2.

ANTIOXIDANT STATUS

Ascorbic acid analysis

Ascorbic acid was measured in EDTA plasma using the method of Vuilleumier and Keck (1989). A detailed description can be obtained by referring to methodology section 3.3.7.1.

α-tocopherol, retinol, lycopene, α- and β-carotene analysis

The HPLC method of Catignani and Bieri (1983) and Thurnham *et al* (1988) was used to stimultaneously determine plasma lipid soluble antioxidant status. A detailed description can be obtained by referring to methodology section 3.3.7.2.

Statistical analysis

Between hour differences were determined using a one-way ANOVA.

Analytical variation assessment

The ***coefficient of variation*** **(CV)** was calculated using the following equation:

$$CV = \frac{SD}{Mean} \times 100$$

A sample was introduced ten consecutive times into the appropriate apparatus to determine the ***coefficient of analytical variation*** **(CV_a).** The stable synthetic radical DPPH was used to determine the analytical precision of the Bruker EMX X-band ESR Spectrometer (*appendix 10*).

Biological variation assessment

Assay data from each subject, collected at periodic times (eight specimens) as previously described was used in the calculation of ***within subject biological coefficient of variation*** **(CV_w)** using the following equation:

$$CV_w\ (\%) = \text{Total variation } (\%) - CV_a\ (\%)$$

All data was tested for the presence of outliers among mean values according to Reeds criterion (Reed *et al*, 1971). This criterion considers the difference between the extreme value and the next highest or lowest value, rejecting the extreme if this difference exceeds one-third the range of all values. All data with outliers excluded are marked *.

Critical difference determination

The ***Critical Difference*** **(CD)** was assessed using the equation of Fraser and Fogarty (1989):

$$CD = K\sqrt{(CV_a^2 + CV_w^2)}$$

Where:

K = factor dependent on the probability level selected (2.77 at $P < 0.05$)

CV_a = *coefficient of analytical variation*

CV_w = *coefficient of within subject variation*

4.2 - RESULTS

(A) Dietary status

Table 4.1 – Nutritional profile (n = 10)

Variable	*Mean + SD*
Energy (Kcal)	2598 ± 264
Fat (grams)	82.6 ± 3.8
Carbohydrate (grams)	298 ± 82.2
Protein (grams)	89 ± 23.4
Fibre (grams)	16.3 ± 7.1
Sugar (grams)	194 ± 33
PUFA (grams)	21.5 ± 3.1
Saturated fats (grams)	34.5 ± 9

Values are means ± SD. Kcal, kilocalories; PUFA, polyunsaturated fatty acids.

Table 4.1 displays the nutritional profile for subjects who participated in the study. These data are within the normal nutritional range for young healthy male individuals (Bender and Bender, 1986).

(B) Physiological parameters

Table 4.2 – The biological variation and critical difference for some physiological parameters over an 8 hr period

Variable	*O_2 uptake*	*Heart rate*	*Haemoglobin*	*Packed cell volume*
CV_a (%)	*0.05*	*0.1*	*0.7*	*0.03*
CV_w (%)	*(1.8 – 8.5)* *4.3*	*(3.2 – 12.1)* *8.2*	*(3.7 – 8.8)* *6.6*	*(2 – 5.5)* *3.5*
True biological variation (%)	***4.25***	***8.1***	***5.9***	***3.47***
Critical Difference (%)	***11.7***	***22.4***	***16.4***	***9.6***

O_2, oxygen uptake; CV_a, coefficient of analytical variation; CV_w, coefficient of within subject biological variation; (within subject biological % range).

Table 4.2 displays common resting physiological parameters that are to be used within studies 1, 2 and 3. Biological variation and critical difference (CD) data are presented, with heart rate having the highest and packed cell volume the lowest CD amongst parameters. The mean resting O_2 consumption was 4.1 ml.kg.min^{-1} or 1.1 mets (*data not shown*).

(C) Oxidative stress parameters

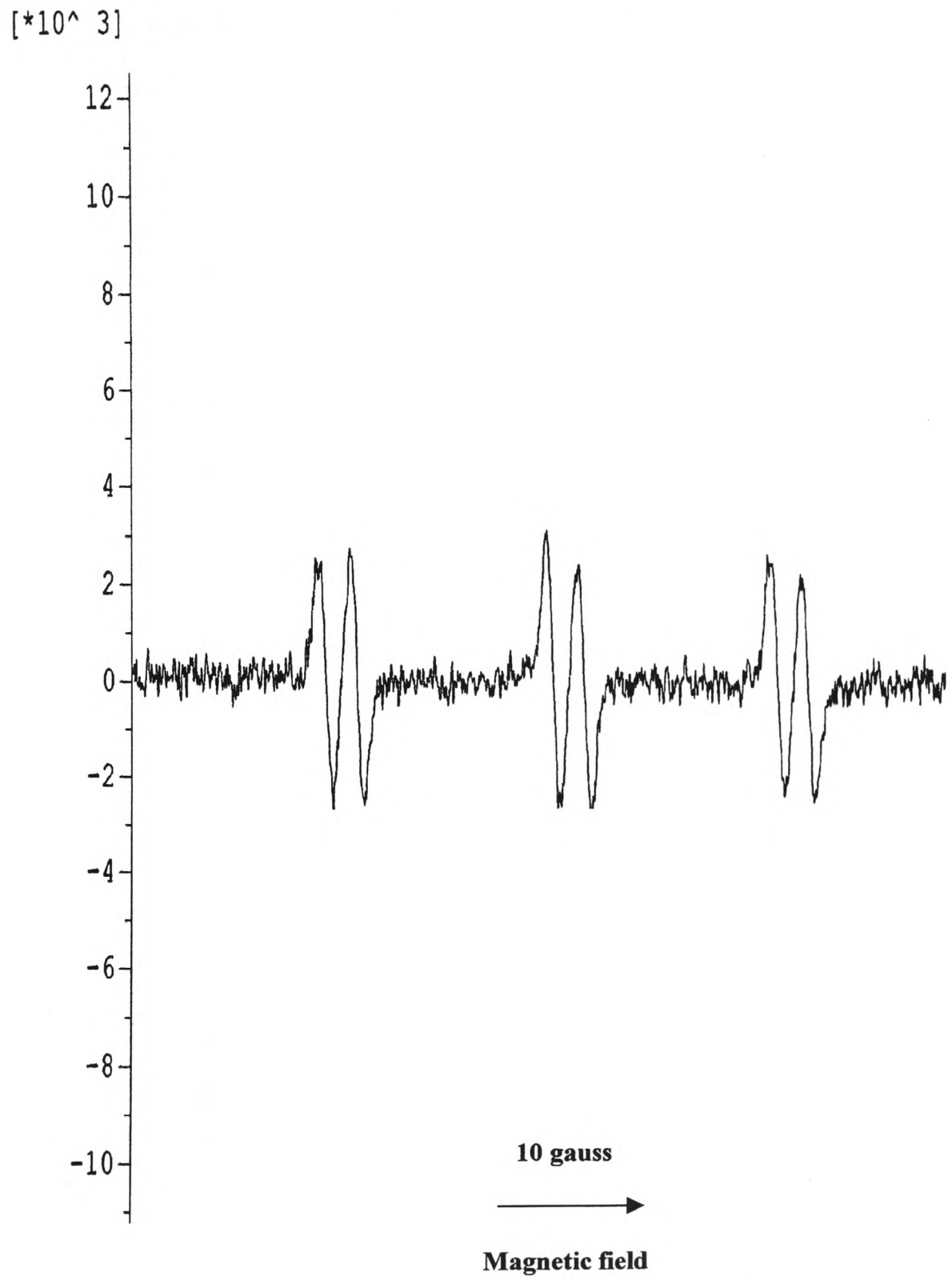
Table 4.3 – The biological variation and critical difference for selected oxidative stress indices over an 8 hr period

Variable	*PBN adduct*	*MDA*	*LH*
CV_a (%)	*5.2*	*4.9*	*4.6*
CV_w (%)	*(26.7 – 71)* *48.6*	*(8 – 58)* *22.4*	*(4 – 43)* *13.4*
True biological variation (%)	***43.4***	***17.5***	***8.8***
Critical Difference (%)	***121***	***50.3***	***27.5***

PBN, α-phenyl-*tert*-butylnitrone; MDA, malondialdehyde; LH, lipid hydroperoxides; CV_a, coefficient of analytical variation; CV_w, coefficient of within subject biological variation; (within subject biological % range).

Table 4.3 summaries the biological variation and CD values for the oxidative stress indices. The PBN adduct recorded the highest biological variation and CD percentage, whilst the lipid peroxidation marker, LH the lowest. MDA had a within subject range difference of 50%, which was the highest recorded, while LH was the lowest at 39%. Blood for PBN adduct analysis only, was also extracted for the determination of within subject variation within a 1 hr period (*every ten minutes = 6 samples*), and subsequently calculated at 70% (n =1) (*data not shown*). Figure 4.1 displays a typical ESR spectra of PBN adduct from human blood. The hyperfine coupling constants of the PBN structure were recorded at a_N 13.7 gauss and $a\beta_H$ 1.7 gauss, suggesting that the free radical species are either peroxyl or alkoxyl, originating possibly from the oxidative modification of lipid hydroperoxides.

Figure 4.1 – Typical ESR spectra of a biological PBN adduct



(D) Antioxidant indices

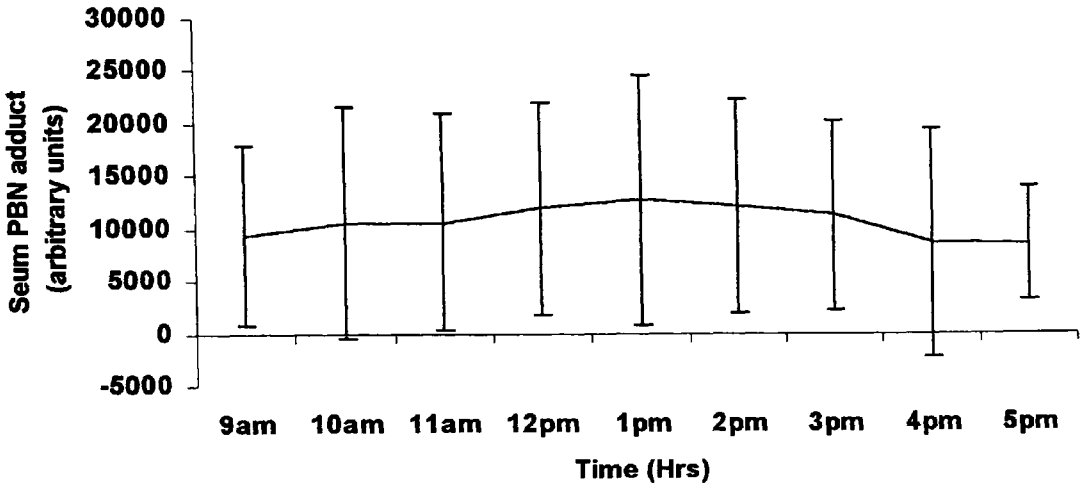
Table 4.4 - The biological variation and critical difference for selected antioxidant indices over an 8 hr period

Variable	CV_a(%)	CV_w(%)	*True biological variation (%)*	*Critical Difference (%)*
Ascorbic acid	*0.68*	*3.7 (1.1 – 8.9)*	***3***	***8.5***
α-tocopherol	*4.6*	*5.6 (2.6 – 16.6)*	***1***	***13***
Retinol	*4.6*	*13.9 (3.9 – 35)*	***9.3***	***28.7***
Lycopene	*4.6*	*42.5 (23.7 – 70)*	***38***	***106***
α-carotene	*4.6*	*43 (21.6 – 78)*	***38.4***	***107***
β-carotene	*4.6*	*13.7 (3.7 – 33.5)*	***9.1***	***28.2***

CV_a, coefficient of analytical variation; CV_w, coefficient of within subject biological variation; (within subject biological % range).

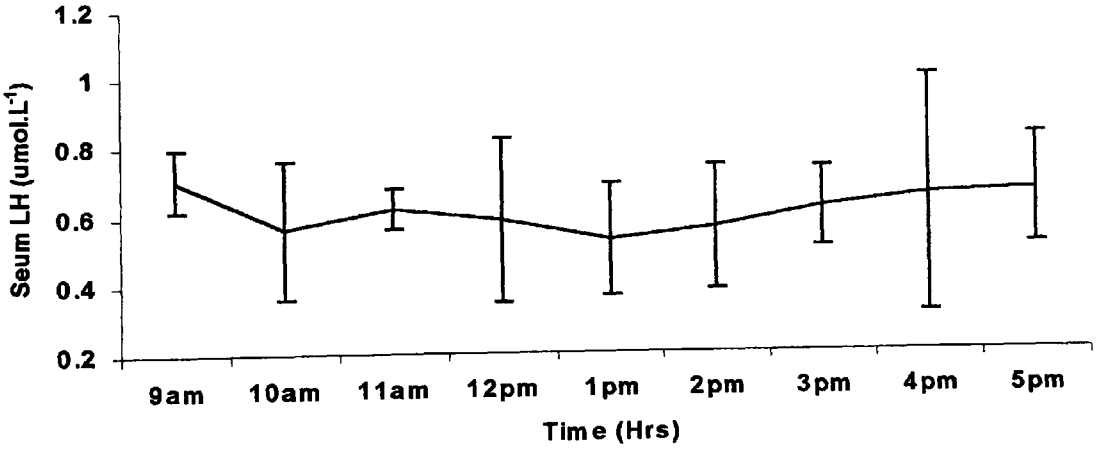
The analytical and biological variation and CD percentages of selected antioxidants are shown in table 4.4. Ascorbic acid, a potent water-soluble antioxidant had the lowest CD percentage, whilst the fat-soluble antioxidant, α-tocopherol possessed the lowest biological variation score. The highest values recorded were from the fat-soluble carotenoids, lycopene and α-carotene.

*Figure 4.2 – Variation for serum PBN adduct over an 8 hr period (*n = 9)*



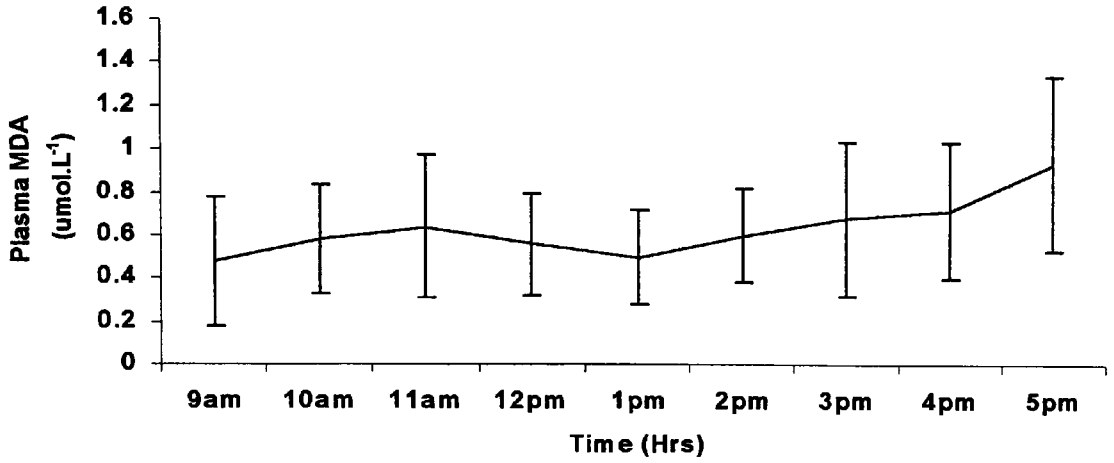
The mean PBN adduct concentration was lowest at 9 a.m. (7759 ± 7729 arbitrary units), and peaked at 1 p.m. (13041 ± 14908 arbitrary units). No difference was observed between any hr ($P > 0.05$).

Figure 4.3 – Variation for serum lipid hydroperoxides (LH) over an 8 hr period



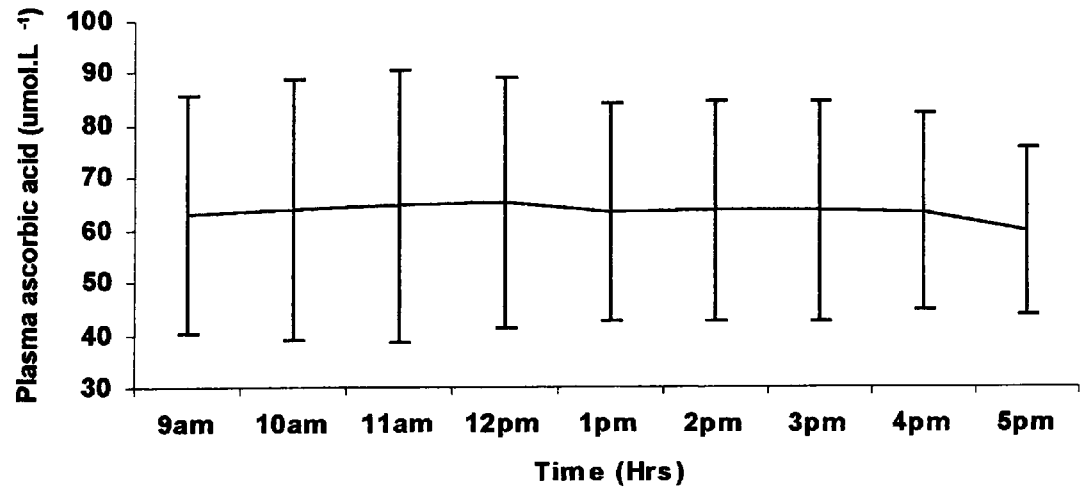
The mean LH concentration was highest at 9 a.m. (0.70 ± 0.09 $\mu mol.L^{-1}$), and lowest at 1 p.m. (0.52 ± 0.16 $\mu mol.L^{-1}$). No difference was observed between any hr ($P > 0.05$).

Figure 4.4 – Variation for plasma malondialdehyde (MDA) over an 8 hr period



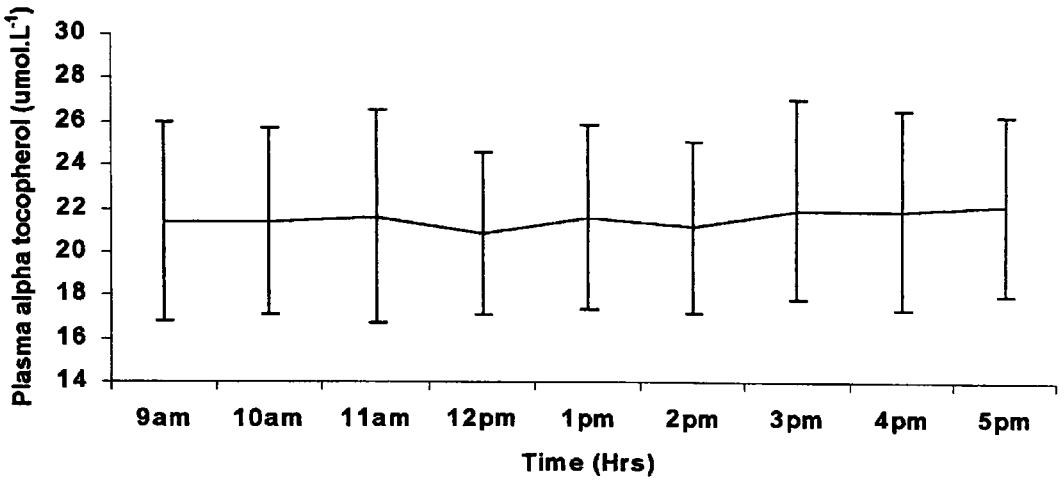
The mean MDA concentration was lowest at 9 a.m. (0.48 $\pm$ 0.30 μmol.L^{-1}), and highest at 5 p.m. (0.94 $\pm$ 0.41 μmol.L^{-1}). No difference was observed between any hr ($P >$ 0.05).

Figure 4.5 – Variation for plasma ascorbic acid over an 8 hr period



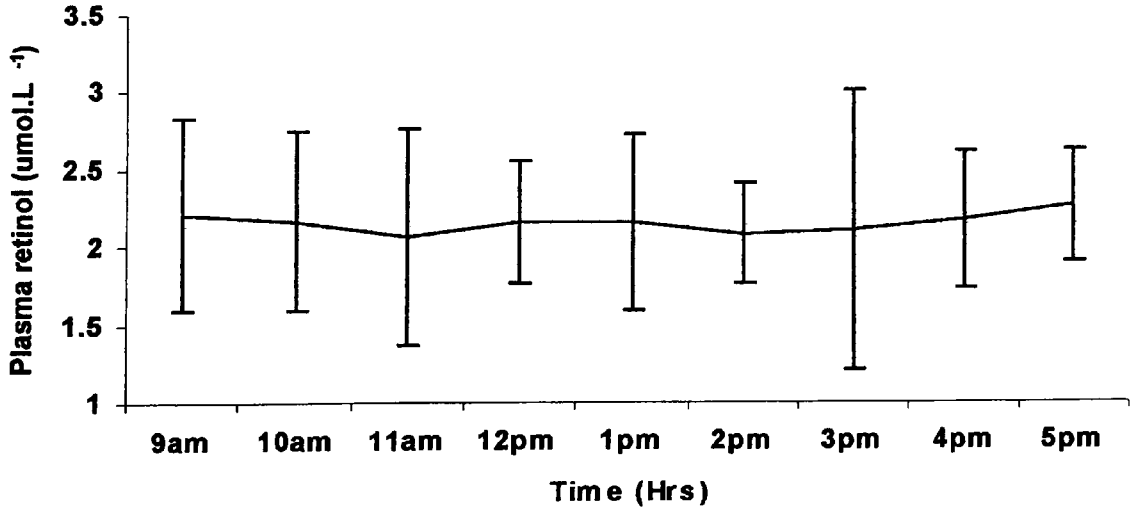
The mean ascorbic acid concentration was highest at 12 p.m. (65.2 $\pm$ 22.6 μmol.L^{-1}), and lowest at 1 p.m. (58.9 $\pm$ 16.7 μmol.L^{-1}). No difference was observed between any hr ($P > 0.05$).

Figure 4.6 – Variation for plasma α-tocopherol over an 8 hr period



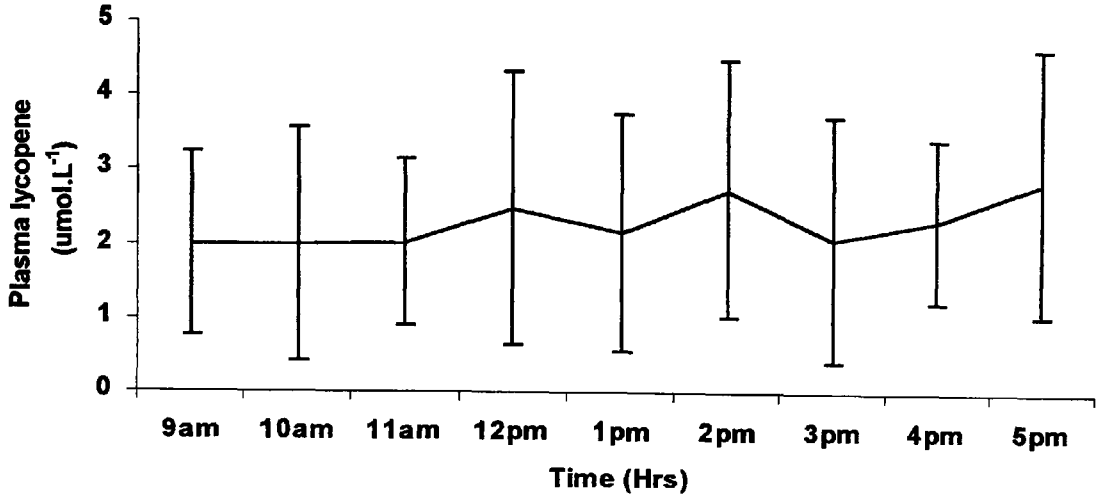
The mean α-tocopherol concentration was lowest at 12 p.m. (20.8 ± 3.7 μmol.L^{-1}), and highest at 5 p.m. (22.2 ± 4.1 μmol.L^{-1}). No difference was observed between any hr ($P > 0.05$).

Figure 4.7 – Variation for plasma retinol over an 8 hr period



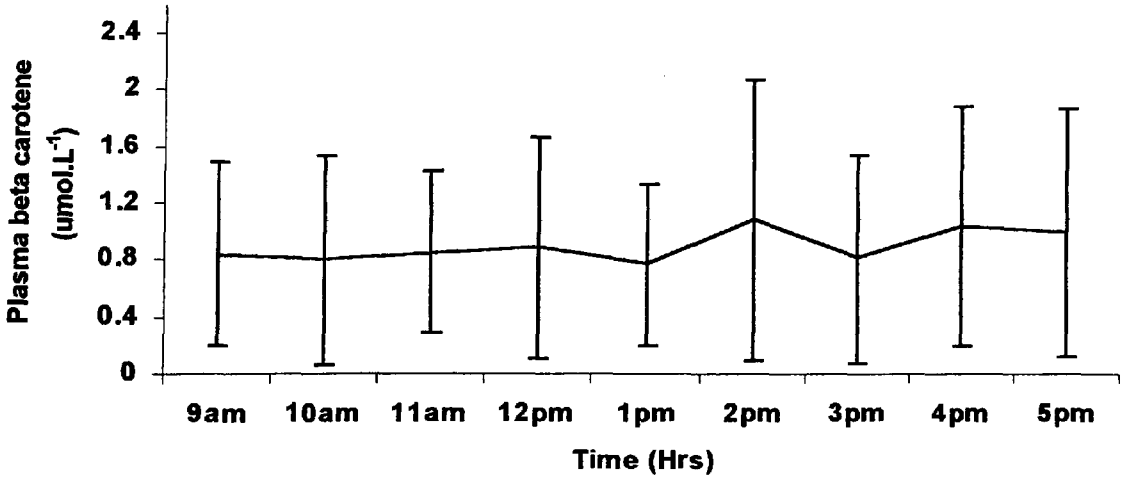
The mean retinol concentration was lowest at 11 a.m. (2 ± 0.7 μmol.L^{-1}), and peaked at 5 p.m. (2.27 ± 0.37 μmol.L^{-1}). No difference was observed between any hr ($P > 0.05$).

Figure 4.8 – Variation for plasma lycopene over an 8 hr period



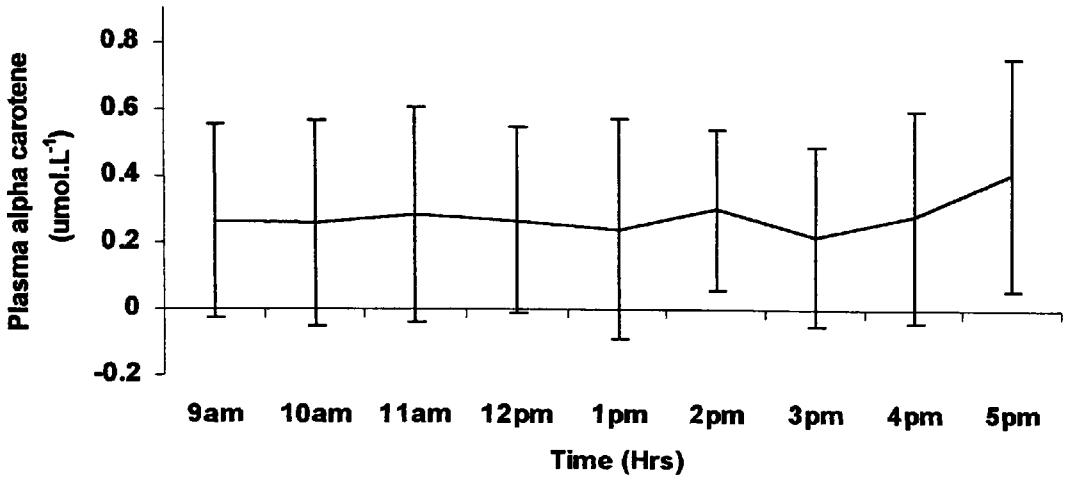
The mean lycopene concentration was lowest at 10 a.m. (1.9 ± 1.6 μmol.L^{-1}), and peaked at 5 p.m. (2.8 ± 2.8 μmol.L^{-1}). No difference was observed between any hr ($P >$ 0.05).

Figure 4.9 – Variation for plasma β-carotene over an 8 hr period



The mean β-carotene concentration was lowest at 1 p.m. (0.77 ± 0.56 μmol.L^{-1}), and peaked at 4 p.m. (1.05 ± 0.85 μmol.L^{-1}). No difference was observed between any hr ($P >$ 0.05).

Figure 4.10 – Variation for plasma α-carotene over an 8 hr period



The mean α-carotene concentration was lowest at 9 a.m. (0.26 ± 0.29 μmol.L^{-1}), and peaked at 5 p.m. (0.41 ± 0.52 μmol.L^{-1}). No difference was observed between any hr ($P > 0.05$).

4.3 – DISCUSSION

The purpose of the present pilot work was to establish the biological variation and critical difference of PBN adduct, lipid peroxidation biomarkers, and selected antioxidants in this group of volunteers. To this authors' knowledge, these are the first data over an 8 hr period of some oxidative stress (*PBN adduct, malondialdehyde and lipid hydroperoxides*) parameters in venous blood. The following section discusses these data; which may contribute towards making conclusions as to whether or not a real physiological difference has been observed in studies 1, 2, and 3.

Oxidative stress components

Little is currently known regarding the biological variation and critical difference of lipid peroxidation *per se*, with only a few studies examining the circadian variation of MDA in humans (Bridges *et al* 1992, Bridges *et al* 1992) and one using animals (Kolosova *et al*, 1983). To date, no attention has been given towards the biological variation and critical difference of free radical species.

This study presents novel data which describes a biological variation of 43.4%, 17.5% and 8.8%, and a critical difference of 121%, 50.3% and 27.5% for the free radical components, PBN adduct, MDA and LH respectively.

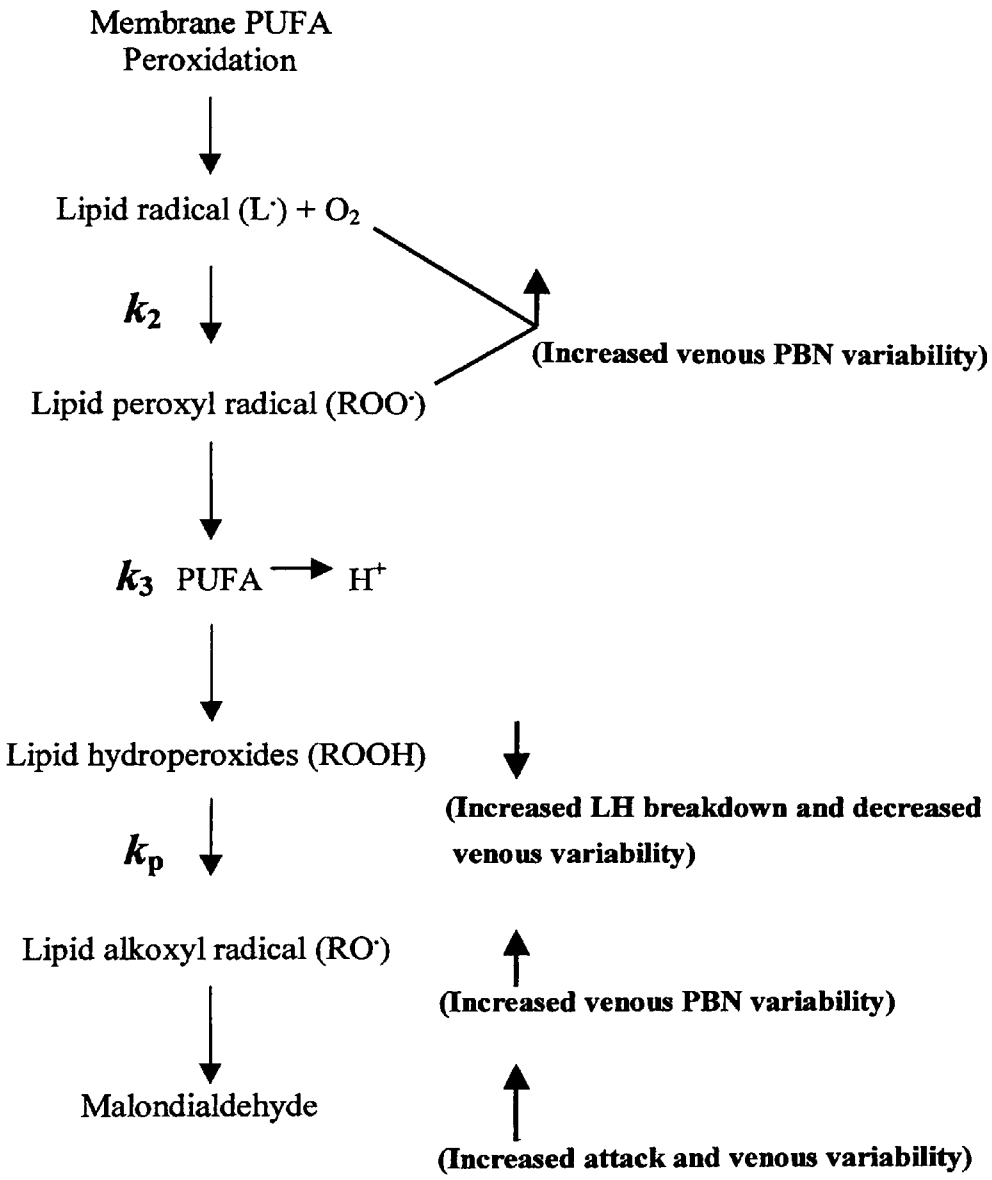
The hyperfine coupling constants for all *ex vivo* PBN trapped radicals observed in the present study were measured at a_N 13.7 gauss and $a\beta_H$ 1.7 gauss, suggesting that the free radical species are either carbon- or oxygen-centred in nature. These coupling constants compare favourably with those of other investigators. For example, Garlick *et al* (1987) observed coupling constants of a_N 13.7 gauss and $a\beta_H$ 1.7 gauss in blood extracted from reperfused and reoxgenated rat heart. These authors suggest, that the nitroxide radical adduct is either a carbon-centred or an alkoxyl radical, both of which may be formed as secondary reactions from initial membrane lipid attack. Mergner *et al* (1991) measured similar hyperfine coupling constants (a_N 13.7 gauss and $a\beta_H$ 2.0 gauss) in PBN and toluene extracted from the venous ischemic, reperfused heart model. These authors also identify the radical as being alkoxyl, and generated from membrane lipid hydroperoxides, formed as a consequence of primary oxygen free radical attack to PUFA. The potent metal chelator desferrioxamine, decreased the free radical concentration detected, confirming the significance of iron in alkoxyl radical formation. Tortolani *et al* (1993) also found comparable coupling constants to the above (a_N 13.6 gauss and $a\beta_H$ 1.9 gauss), in coronary sinus blood from patients undergoing elective cardioplegia. The radicals were identified as characteristic PBN alkoxyl adducts, formed secondarily in the extracellular compartment by iron dependent decomposition of lipid hydroperoxides. Interestingly, these investigators also detected the presence of a second species with a much larger β hydrogen split (a_N 14.1 gauss and $a\beta_H$ 4.2 gauss). This species was identified as being a carbon-centred radical contributing to 17% of the overall radical concentration as recognised from the signal amplitude.

The presence of a mixture of carbon and oxygen-centred radical adducts of PBN was also previously reported in patients following percutaneous transluminal coronary angioplasty (Coghlan *et al*, 1991). Therefore, since both oxygen and carbon-centred radical adducts can be detected within the same or near hyperfine coupling constant range, caution is required when attempting to categorically attribute coupling constants to a particular radical species. Accurate radical identification may only be attained by

the use of more specific spin traps (*e.g.* d_{14}PBN) and through the use of ENDOR spectroscopy, which results in much narrower line widths (Davies and Timmins 1996, Forman and Borg 1989, Thornalley 1986). Unfortunately ENDOR spectroscopy is not applicable to biological samples due to a 10-fold decrease in sensitivity and to the low concentration of radicals in biological samples (*Personal communication, Dr T Ashton*). Thus, the author tentatively suggests that the species detected in the present study may be either alkoxyl, peroxyl or a combination of both lipid radicals, or the alkyl radical, formed from the β-scission and decomposition of the alkoxyl radical. It would be difficult to argue against the sole detection of the peroxyl radical, since its half-life is much greater (*i.e.* 7 secs, Pryor, 1986) than the alkoxyl radical (1×10^{-6}, Pryor, 1986). However, the PBN-peroxyl adduct is generally not thought to be stable enough for room temperature detection, whereas the PBN-alkoxyl spin adduct is a more stable compound at room temperature (Mason, 1984). Further work is required to differentiate and confirm the exact species detected.

In agreement with the previous authors (Garlick *et al* 1987, Mergner *et al* 1991, Tortolani *et al* 1993), it is suggested that the lipid radicals detected in the present study, are formed as a result of PUFA and lipid hydroperoxide decomposition, and are possibily secondary or tertiary radicals, formed well "downstream" the lipid peroxidation chain of events (*figure 4.11*). Furthermore, the author suggests that the radicals detected may be a consequence of primary radical attack to PUFA rich erythrocyte membranes or circulating lipids, initiating PUFA breakdown which would yield radical intermediates from lipid oxidation (Clemens and Waller 1987, Duthie 1993). This implies that the systemic circulation is the primary location of lipid peroxidation and is supported by evidence from Dickens *et al* (1991) who detected alkoxyl radicals (a_N 13.7 gauss and $a\beta_H$ 2.2 gauss) in endothelial cells on re-oxygenation. In support, Bolli *et al* (1989) report secondary oxygen- or carbon-centred radical detection using PBN, but more importantly suggested that the species are either alkoxyl or alkyl radicals formed as a result of initial oxygen-centred radical attack to membrane lipids. Likewise, Hartell *et al* (1994) confirmed the presence of carbon-centred lipid radicals with hyperfine constants in the range of a_N 13.5-13.7 gauss and $a\beta_H$ 2.0-2.2 gauss, and suggested that these radicals occur as a result of lipid membrane attack by primary oxygen centred species.

Figure 4.11 – Event sequence within lipid peroxidation cascade



PUFA, polyunsaturated fatty acids; H^+, hydrogen atom, L·, lipid radical; O_2, oxygen molecule. *K_2, K_3 and K_p* = reaction kinetics.

The availability and concentration of fatty acid molecules *in vivo,* is paramount to the detection of *ex vivo* PBN trapped lipid radicals. Although the components of total lipids were not measured in the present study, the hypothesis that the PBN adducts detected are formed primarily as a result of fatty acid breakdown, is strengthened by a similar biological variation percentage of fatty acid components, as shown from other published work. Demacker *et al* (1982) observed a 35.7% triglyceride biological variation during a 1-day protocol in 28 hospital workers. Significantly, a similar range (6.3-65%) to the PBN adduct in the present study was observed. Other investigators report similar triglyceride biological variation percentages (32.7%, Evans and Laker 1995; 41.2%, Costongs *et al* 1985; 20%, Widjaja *et al* 1999).

It is pertinent at this stage, to draw attention to the fact the subjects in this study were fasted throughout the experimental period. It is suggested that as the day progressed, the predominant energy source was from an increased utilisation of fatty acid molecules derived from adipose tissue sites (Randle *et al* 1963, Guyton and Hall 2000).

Since the mean dietary intake of PUFAs in the present data were within normal limits (Bender and Bender, 1986), it may be speculated that as the day went on an increase in circulating fats would provide more substrate within the systemic circulation for oxidation. This is supported by the work of Anderson *et al* (2001) who has shown increased detection of alkoxyl radicals following a high fat meal.

Coghlan *et al* (1991) has stated that "*lipid hydroperoxides, the primary molecular products of lipid peroxidation, are produced by the reaction of free radicals with cholesterol or PUFAs followed by an interaction of the primary carbon-centred radical with oxygen*". The biological variation of the PBN adduct (43.4%) observed in the present study may be partially due to the rapid decomposition of lipid hydroperoxides, since it is widely accepted that alkoxyl radicals are generated from the breakdown of lipid hydroperoxides (Mergner *et al* 1991, Tortolani *et al* 1993), as previously shown by Yue Qian *et al* (2000). Additionally, Coghlan *et al* (1991) detected PBN trapped carbon-centred radicals in the presence of lipid hydroperoxides in blood samples from patients undergoing coronary angioplasty. In support of this hypothesis, the biological variation of lipid hydroperoxides observed in the present study was much lower at 8.8% in comparison with the PBN adduct variation.

Vladimirov *et al* (1980) offers various mechanistic suggestions as to what controls the decomposition of lipid hydroperoxides in cellular membranes, these include; oxidised (Fe^{2+}) and reduced iron (Fe^{3+}), oxygen, lipid antioxidants and reaction rate constants.

The most important of the above in this authors opinion are the components of iron. Lipid hydroperoxides depend on the presence of localised Fe^{2+} and Fe^{3+} to generate alkoxyl radicals (*figure 2.6, p32*), therefore, in the absence of oxidised iron, the lipid peroxidation cascade would inevitably terminate before the formation of the tertiary free radical species. Unfortunately iron was not accounted for in the present study, however, Costongs *et al* (1985) have previously reported a biological variation of 22.3% for serum iron, of which incidentally, only small amounts of iron are required to breakdown hydroperoxides and give rise to a free radical flux (Coghlan *et al*, 1991). Moreover, iron is loosely bound to transferrin and can become *unbound* at any point in time (Guyton and Hall, 2000). Due to the detection of the alkoxyl radical observed in the present study, it is postulated that Fe^{2+} is prominent in the rapid degradation of lipid hydroperoxides, yielding a much lower biological variation than that observed for the PBN adduct. In support of the importance of iron in lipid hydroperoxide decomposition, Lai and Piette (1977) investigated adducts produced in the presence of $OH^{\cdot}$ radicals generated under UV irradiation of a 1% H_2O_2 solution. The observed EPR signal was attributed to the adduct formed on binding of the $OH^{\cdot}$ radical to PBN, however, when Fe^{2+} was added to the medium, the adduct exhibited quite different EPR spectra reflecting the different nature of radical detected. In providing further support, it has been demonstrated that by adding Fe^{2+} to mitochondrial suspensions, there is rapid hydroperoxide decay (Vladimirov *et al*, 1980).

Furthermore, Vladimirov *et al* (1980) suggests that the rate of lipid peroxidation breakdown is controlled by the concentration and reaction rates of the carbon-centred lipid radical ($L^{\cdot}$) and the oxygen-centred peroxyl radical ($ROO^{\cdot}$), thus an increase in the reaction rate constants k_2 and k_3 as shown in figure 4.11, would inevitably increase lipid hydroperoxide decay as shown in *k*p. The reaction rate constant for k_p is estimated at approximately 3.2×10^2 m^{-1} sec^{-1} based on controlled amounts of PUFA, and will however, increase in the presence of iron (Vladimirov *et al*, 1980). Therefore, the higher biological variation of the free radicals detected in the present study may be

significant in contributing towards speeding up the reaction rates within the lipid cascade, and in doing so decreasing the biological variation of lipid hydroperoxides.

It is also imperative to note that the speed of these reactions will increase as more PUFAs are presented for oxidation (Vladimirov *et al*, 1980). Therefore, the previous hypothesis outlined, indicating alkoxyl radical formation from increased PUFAs breakdown may be apparent here.

The present study describes a biological variation of 17.5% for MDA, somewhat lower than the PBN adduct variation and slightly higher than the biological variation for lipid hydroperoxides. Since MDA is formed primarily by alkoxyl radical attack within the lipid peroxidation cascade (Janzen, 1980), it is suggested that the detection of the PBN adduct in the present study contributed towards MDA formation (*figure 4.11*). This is supported by the low biological variation of the main lipid soluble antioxidants, α-tocopherol and β-carotene, thus, the lack of potent antioxidant variation (*decreased scavenging capacity*) in the venous circulation would inevitably allow the alkoxyl radicals to progress further within the cascade, and in so doing produce MDA. However, some degree of radical scavenging and protection is offered, as apparent by the much larger biological variation of the lipid soluble antioxidants, lycopene and α-carotene. Additionally, it had been shown that by using PBN as an antioxidant to intercept the lipid oxidation cascade, the production of MDA may be attenuated (Saprin and Piette, 1977).

Due to the plethora of potential reactions with cellular components, accurate estimation of how much radical is reacting with other components of the biological system is virtually impossible. For example, the present investigation concentrated solely on the by-products of lipid oxidation, thus, other vital information may have been obtained by examining the by-products of DNA oxidation.

Moreover, in such an intricate biochemical system as venous blood, it is virtually impossible to determine the origin of the initiating primary radical species. The ESR detection of the primary species (*e.g.* $O_2^{\cdot-}$ or/and $OH^{\cdot}$) is extremely difficult in biological fluid due to the fast interaction of these highly charged species with surrounding

biological molecules (Finkelstein *et al*, 1980). For example, a reaction rate constant of 10^8 $M.s^{-1}$ has been reported for the hydroxyl radical (de Groot, 1994). Therefore, the inability to detect primary species in biological fluid, perhaps, confirms the suggestion that the radicals detected in the present study are alkoxyl radicals, possibly derived from lipid membrane peroxidation by primary oxygen-centred species (Ashton, 1998).

Much evidence has accumulated towards the mitochondria as a primary source of $O_2^{\cdot-}$ $OH^{\cdot}$ and H_2O_2 generation (Boveris and Chance 1973, Boveris and Cadenas 1975), in addition to much speculation that increased mitochondrial O_2 flux may increase ROS generation. Since previous suggestions imply that the systemic circulation is the probable location of lipid peroxidation, the mitochondria can be categorically ruled out as a potential source in the present study, due to the lack of mitochondrion present within erythrocytes (Tortora and Grabowski, 1996), and perhaps more importantly, the low O_2 consumption as evident within the present data. However, if cellular mitochondria were involved at any level, it is expected that the complex intracellular antioxidant network would immediately scavenge and control any free radical generation at rest.

The author presents one potential mechanistic source of primary oxygen-centred radical generation, which could be involved in the apparent attack to lipid molecules, as evident by the detection of lipid radical species and lipid hydroperoxides/malondialdehyde in the present study.

This potential source involves the auto-oxidation of various blood metabolites. Theoretically, any physiological molecule with an electron to donate can be oxidised, however, current literature is primarily concerned with glucose, catecholamine and haemoglobin molecules as contributors of $O_2^{\cdot-}$ (Young and Woodside 2001, Svistunenko *et al* 1997). It is postulated that auto-oxidation of these molecules will generate $O_2^{\cdot-}$ which initiates the process of lipid peroxidation, yielding lipid radicals for detection (Halliwell and Gutteridge, 1999). Even in the presence of the antioxidant enzyme SOD in the extracellular compartment, lipids may still come under attack by the subsequent formation of H_2O_2, and then $OH^{\cdot}$ radical generation through an iron dependent reaction. Recent EPR evidence has been presented confirming haemoglobin autoxidation *in vivo* (Svistunenko *et al*, 1997).

Unfortunately, the biological variation of glucose or catecholamines was not accounted for in the present study. However, the biological variation of haemoglobin was determined as being 5.9%. What this means in terms of the biological variation of the oxidative stress components has yet to be established.

Antioxidant components

The present study shows a biological variation of 3%, 1%, 9.3%, 9.1%, 38% and 38.4% for the antioxidants, ascorbic acid, α-tocopherol, retinol, β-carotene, lycopene and α-carotene respectively.

Gallagher *et al* (1992) report similar values for retinol (11.3%) and β-carotene (15.6%), whilst contrasting data are reported for the main lipid and aqueous antioxidants, α-tocopherol (17.4%) and ascorbic acid (15.4%) respectively. Since the analytical methods of antioxidant determination were the same in the present study as that of Gallagher *et al* (1992), the only other explanation is perhaps a greater variance in subject antioxidant consumption existed in the study by Gallagher *et al*, as there was no mention of excluding individuals supplementing with antioxidants prior to the experimental phase. In agreeing with the present data, Maes *et al* (1996) display virtually identical α-carotene (38.3%) and retinol (8.9%) values in twenty apparently healthy Belgian men. In comparison, a larger value for ascorbic acid (18.2%) is given, which may reflect a difference in the sensitivity of the various analytical methods employed.

The biological variation of the selected antioxidant indices may be separated into two sections, (1) antioxidants with a low biological variation (*ascorbic acid, α-tocopherol, retinol, β-carotene*), and (2) those with a higher biological variation (*lycopene and α-carotene*).

The former section consisting of the main lipid and aqueous phase antioxidants α-tocopherol and ascorbic acid, may provide a reason for the biological variation in the PBN adduct. The low variation within these antioxidants is clearly not sufficient to counteract and control the variation in lipid radicals generated. It is suggested that the

lipid antioxidants are pre-occupied with defending cellular membrane structures (Brigelius-flohe and Traber, 1999) which are regularly attacked by oxygen radicals. The low within subject variation for ascorbic acid may not necessarily reflect inadequate dietary intake, but the ability to help scavenge the radicals detected. Ascorbic acid has been termed, an outstanding antioxidant in human blood plasma (Frei *et al*, 1989), and is known to be an excellent scavenging antioxidant particularly within an aqueous environment (Frei, 1994).

The latter section contains two less potent antioxidants than the above, nevertheless, the biological variation of these antioxidants match that of the PBN adduct variation, suggesting a possible biochemical response to help combat the wide variation of lipid free radicals.

4.4 – CONCLUSION

The purpose of the present work was to establish preliminary data on the biological variation and critical difference of the PBN adduct, measures of lipid peroxidation and individual antioxidants in human blood. To this authors knowledge these are the first oxidative stress data of its kind.

The biological variation of selected biochemical parameters relevant to this thesis have been outlined in previous sections, and suggestions offered as to why there is such a variation between the oxidative stress indices.

The critical difference of the PBN adduct, MDA and LH was estimated at 121%, 50.3% and 8.8% respectively. As the critical difference effectively stems from the biological variation, little attention has been given to its explanation throughout this chapter, however, it is hoped to be incorporated in the forthcoming chapters to determine whether or not exercise and pathology *per se* contribute towards a *real* physiological change.

Furthermore, the critical difference in the present study was calculated using biochemical data from *only* apparently healthy male individuals. Data on biological variability and critical difference in individuals with a known pathology have not been

included in this study, and have yet to be determined. However, the existing data will provide an approximation for the pathological population examined in studies 2 and 3 of the present thesis.

Chapter 5

Study 1

Exercise and Oxidative Stress During Prolonged Exercise in Normobaric Hypoxia

5.0 – INTRODUCTION

During aerobic physical exercise oxygen utilisation is increased which may lead to the incomplete reduction of oxygen molecules in one or more mitochondrial complexes, resulting in an extensive increase in free radical generation (Rokitzki *et al* 1994, Mohanraj *et al* 1998). This phenomenon, which is associated with increased levels of lipid, protein and DNA oxidation, is collectively termed *oxidative stress* and occurs not only in physically active healthy individuals, but is also related be it causal or consequential to the pathology of human disease (Halliwell 1994, Ashton *et al* 1998). Oxidative stress can occur during physical exercise not only via the mitochondria as a primary source, but from other mechanisms such as substrate auto-oxidation, xanthine oxidase activity, neutrophil activation, nitric oxide synthesis and metal catalysed reactions (Sen *et al*, 2000).

Paradoxically, a growing body of evidence suggests that exercise performed in hypoxia may also stimulate oxidative stress due primarily to a decrease in mitochondrial respiration and a build-up of reducing equivalents that cannot be transferred to molecular oxygen at the level of cytochrome oxidase (Simon-Schnass, 1994). In addition to this emerging concept known as reductive stress, other potential sources of free radical generation in hypoxia include, increased nitric oxide production, xanthine oxidase and phospholipase A_2 activation, and increased availability of free Fe and Cu (Mohanraj *et al* 1998, Bailey *et al* 2001, Kanter 1998). The influence of hypoxia on lipid peroxidation has been explored by a numbers of investigators (Radak *et al* 1994, Simon-Schnass 1994, Vasankari *et al* 1997, Nakanishi *et al* 1995), and research from our laboratory (Bailey *et al*, 2000) has observed an increase in lipid hydroperoxide and malondialdehyde production following maximal exercise in normobaric hypoxia. Other recent work by Bailey *et al* (2001) has shown that physical exercise training in intermittent hypoxia can attenuate the exercise-induced increase in these putative biomarkers of lipid peroxidation more effectively than normoxic training, suggesting that hypoxic exercise may have influential molecular adaptive properties. However, a major criticism of the these studies is that only indirect indices of free radical-induced molecular damage were used, thus the claim that hypoxia generates free radicals species has not been confirmed in the exercising human. To date, Electron Spin Resonance (ESR) spectroscopy in conjunction with the spin trap technique has only been used to

confirm the presence of free radical species in the venous blood of humans exercised to physical exhaustion (Ashton *et al* 1998, 1999). To this authors knowledge there are currently no studies that have used ESR spectroscopy to directly determine the pro-oxidant effects of prolonged aerobic exercise performed in normobaric hypoxia. Therefore the purpose of the present investigation was to quantify the degree of free radical mediated oxidative stress in acute normobaric hypoxic exercise. A further aim was to validate ESR spectroscopy as a direct measure of free radical production in prolonged aerobic exercise by comparing signal changes with conventional lipid peroxidation markers.

5.1 – METHODOLOGY

Subjects

Thirty (n = 30) apparently healthy male volunteers were recruited from the student population of the University of Glamorgan to participate in the present study (*table 5.1 for subject characteristics*). All subjects were physically active and performed between 1 to 2 h week^{-1} of individual or team sport. Subjects had no known physician diagnosed diseases or ailments assessed by a medical history questionnaire prior to experimental exercise. All subjects were non-smokers and any subjects who took antioxidant supplements were excluded.

Experimental design

The local Medical Research Ethics Committee (Bro Taf) granted ethical approval and written informed consent was obtained from each subject prior to participation. Subjects were instructed to abstain from any dietary antioxidant supplementation for six weeks prior to experimental exercise. Subjects were instructed to refrain from exercise and alcohol for 48 h before all tests, and to maintain their usual dietary pattern up until the last meal consumed 12 h before experimental exercise, where a standardised meal replacement (Wake-up cereal, Retail Brands, London, UK) drink was issued to all subjects (*table 5.0 for nutritional content*). 0.32g of powder per kg of body weight was mixed with 2.7 ml of semi-skimmed milk per kg of body weight by the same investigator. Dietary composition and caloric intake in the 72 h before the exercise test was recorded by means of a food diary and assessed using a standard nutritional

assessment package as described in methodology section 3.11. All subjects attended a familiarisation session 1-week prior to the commencement of the incremental test protocol.

Table 5.0 – Nutritional content per 100 grams

Variable	*Content*
Energy (KJ/kcal)	1480/355
Carbohydrate (g)	79.2
Fat (g)	1.1
Protein (g)	5.4
Vitamin C (mg))	61.6
Vitamin E (mg)	10.3

Anthropometric measures

On arrival at the laboratory, subject body mass and stature was determined as outlined in the methodology section 3.4.

Body fat assessment

Body fat was determined according to the method of Durnin and Womersley (1974). A detailed description can be obtained by referring to methodology section 3.4.1.

Normobaric hypoxic chamber

The same normobaric hypoxic chamber was used for all tests. A detailed description can be obtained by referring to methodology section 3.7.

Oxygen uptake ($\dot{V}O_2$) assessment

$\dot{V}O_2$ was monitored using an off-line gas analysis system (Douglas bag) as described in methodology section 3.6.2.

Heart rate (HR) and rate of perceived exertion (RPE) assessment

HR and RPE were determined using an ECG device and a borg scale as described in methodology sections 3.5.1 and 3.9 respectively.

Oxygen saturation (SaO_2)

SaO_2 was determined using a pulse oximeter (placed on middle index finger) as previously described in methodology section 3.5.2.

Pilot studies

A series of pilot tests were conducted prior to subject participation in order to determine the chamber hypoxic inspired fraction. A subject not involved in the main study participated in all pilot work completed (*appendix 4*).

Incremental test protocol

All incremental exercise tests were performed between 9 a.m. and 5 p.m. at the University of Glamorgan, under the supervision of the same investigators to help control for inter analytical subject variation.

Each subject in a randomised, double-blind placebo-controlled fashion, performed two incremental cycling tests to volitional exhaustion in normobaric normoxia [inspired fraction of oxygen (F_IO_2) of 20.9%] and normobaric hypoxia (F_IO_2 = 16%). Each test was separated by 7 days. Each ergometer cycling (*section 3.8*) test adopted the following protocol:

- 5 min warm up period at 0.5 kg at 80 rpm
- The subject was required to maintain 80 rpm while 0.4 kg was applied to the basket every 2 minutes (power output increase of 32 W.stage^{-1}) until volitional exhaustion.

This protocol was chosen as it had previously been validated to elicit $\dot{V}O_{2\,max}$ in a hypoxic environment (Engfred *et al*, 1994). Pre and post-exercise blood lactate was measured according to the procedures outlined in methodology section 3.3.13. Validation of $\dot{V}O_{2\,max}$ was obtained if whole blood lactate concentration was > 9 mmol.L^{-1} and a heart rate value to within 10 b·min^{-1} of age predicated maximum (220 bpm-age) was achieved. $\dot{V}O_2$ was monitored during the last 60 s of each stage and at the point of volitional exhaustion. HR was continuously recorded throughout. The relationship between $\dot{V}O_2$, power output (*workload max*) and HR was subsequently determined for each subject and used in assessing the individual exercise intensity level for the 2 h protocol. Handle bar angle and seat height was recorded for each subject and repeated in the following protocol.

Experimental protocol

All 2 hr exercise tests were performed between 8 a.m. and 1 p.m. at the University of Glamorgan. Subjects were randomly matched according to their normobaric normoxic $\dot{V}O_{2peak}$ (*table 5.1*), determined in the previous test protocol outlined, and randomly assigned to 1 of 3 groups.

Group 1 – (Norm): Subjects (n = 10) performed 2 h of cycling exercise in normobaric normoxia (F_IO_2 = 20.9%) at a workload corresponding to 55% of the pre-determined $\dot{V}O_{2peak}$ in normobaric normoxia.

Group 2 – [Hyp (rel)]: Subjects (n = 10) performed 2 h of cycling exercise in normobaric hypoxia (F_IO_2 = 16%) at a workload corresponding to 55% of the pre-determined $\dot{V}O_{2peak}$ in normobaric hypoxia.

Group 3 – [Hyp (abs)]: Subjects (n = 10) performed 2 h of cycling exercise in normobaric hypoxia (F_IO_2 = 16%) at a workload corresponding to 55% of the pre-determined $\dot{V}O_{2peak}$ in normobaric normoxia.

$\dot{V}O_2$ was monitored during the last 60 sec of each 10 min period. HR, RPE and SaO_2 were recorded at selected intervals throughout. The laboratory was regulated for temperature (21 ± 2°C) and humidity (65 ± 3%) as described in methodology section 3.10.

Table 5.1 – Subject characteristics

Group	*Norm (n = 10)*	*Hyp (rel) (n = 10)*	*Hyp (abs) (n = 10)*
Age (yrs)	21.4 ± 1.3	21.2 ± 1.3	21.7 ± 2
Stature (cm)	176 ± 5.7	173 ± 4	178 ± 4.3
Body mass (kg)	75 ± 13	72.4 ± 8	73.18 ± 9
Body mass index (kg/m^2)	23.86 ± 3.6	23.92 ± 2.25	22.7 ± 18.2
Skin folds	47 ± 18.5	39.6 ± 21.4	42.4 ± 4
Bodyfat (%)	17.4 ± 4.5	15 ± 6	15.8 ± 5
$\dot{V}O_{2peak}$ ($ml.kg.min^{-1}$)	46.7 ± 5.6	48.1 ± 6	48.6 ± 9

Values are means ± SD. All groups were equally matched for the above characteristics. $\dot{V}O_{2peak}$ ($ml.kg.min^{-1}$), peak oxygen uptake per min.

Venous blood sampling

Pre- and during-exercise blood samples were collected in the experimental protocol only following a 12 hr overnight fast using the cannula method as outlined in methodology sections 3.3.1 and 3.3.2.

PBN adduct preparation and analysis

Fresh PBN was prepared in the morning of experimental day. The PBN adduct was analysed and the spectra measured on a Bruker EMX X-band ESR spectrometer. A

detailed description of PBN preparation and adduct analysis can be obtained by referring to methodology section 3.2.7.

Neutrophil and leukocyte analysis

Whole blood leukocytes and neutrophils were analysed using an automated haematology analyser. A detailed description may be obtained by referring to methodology section 3.3.9.

LIPID PEROXIDATION MEASURES

Malondialdehyde (MDA) analysis

MDA was measured by HPLC in EDTA plasma using a modified method of Young and Trimble (1991). A detailed description can be obtained by referring to methodology section 3.3.6.1.

Lipid hydroperoxide (LH) analysis

LH was measured spectrophotometrically in serum using a modified method of Wolff (1994) and Nourooz-Zaheh *et al* (1994) (***F***errous ***O***xidation of ***X***ylenol orange method; FOX 1). A detailed description can be obtained by referring to methodology section 3.3.6.2.

ANTIOXIDANT STATUS

Ascorbic acid analysis

Ascorbic acid was measured in EDTA plasma using the method of Vuilleumier and Keck (1989). A detailed description can be obtained by referring to methodology section 3.3.7.1.

Retinol, α-tocopherol, β-carotene, α-carotene and lycopene analysis

The HPLC method of Catignani and Bieri (1983) and Thurnham *et al* (1988) was used to stimultaneously determine plasma lipid soluble antioxidant status. A detailed description can be obtained by referring to methodology section 3.3.7.2.

Magnesium analysis

Magnesium was determined in whole blood by dry chemistry slide technology. A detailed description can be obtained by referring to methodology section 3.3.10

MUSCLE DAMAGE MARKERS

Total phosphocreatine kinase analysis

Creatine kinase was measured using the Kodac Ektachem clinical chemistry slide method. A detailed description can be obtained by referring to methodology section 3.3.8.1

Myoglobin analysis

Myoglobin was analysed using the Chiron Diagnostics ACS:180 Automated Chemiluminescene System. A detailed description can be obtained by referring to methodology section 3.3.8.2.

Plasma volume assessment

Blood haemoglobin and packed cell volume were measured to calculate the change in plasma volume (Dill and Costill, 1974; refer to methodology sections 3.3.4 and 3.3.5).

Statistical analysis

Statistical analysis was performed using the SPSS social statistics package - version 9.0 (Surrey, UK). Data were analysed using parametric statistics following mathematical

confirmation of a normal distribution by repeated Kolmogorov-Smirnov tests. For a detailed description of statistical procedures refer to methodology section 3.12.3.

5.2 – RESULTS

(A) Dietary status

Table 5.2 – Nutritional profile (n = 30)

Variable	*Normoxia*	*Hypoxia (Rel)*	*Hypoxia (Abs)*
Energy (Kcal)	2018 ± 456	2092 ± 378	2040 ± 277
Carbohydrates (%)	51.7 ± 4	51.6 ± 5.2	52.1 ± 4.7
Total fat (%)	32.2 ± 3.8	33.3 ± 5.1	32.6 ± 3.5
Saturated fat (%)	11.8 ± 2.1	12.1 ± 3	12.7 ± 2.2
Protein (%)	16 ± 3	15 ± 2	15.3 ± 2.7
Fibre (g)	14.3 ± 6.4	17.4 ± 3	15 ± 2.6

Values are means ± SD. Kcal, kilocalories.

There was no significant difference in caloric intake and macronutrient composition between groups, and all values were within the recommended UK daily range (Bender and Bender, 1986).

(B) Incremental exercise tests

Table 5.3 – Peak O_2 uptake ($ml.kg.min^{-1}$) scores in normoxia and hypoxia

Condition	*Normoxia*	*Hypoxia*
Norm (n =10)	46.7 ± 5.6	28 ± 5.4
Hyp (rel) (n = 10)	48.17 ± 5.8	29.8 ± 6
Hyp (abs) (n = 10)	48.58 ± 9.5	32.1 ± 11.2

Table 5.3 demonstrates the mean O_2 uptake at peak exercise for both conditions. There was a main effect for condition (*normoxic vs. hypoxic, $P < 0.05$*), however, no interaction effect was observed (*condition x group, $P > 0.05$*). This data implies that maximal exercise in hypoxia decreases systemic oxygen concentration.

Table 5.4 – Peak workload (kg) scores in normoxia and hypoxia

Condition	*Normoxia*	*Hypoxia*
Norm	3.5 ± 0.3	3.3 ± 0.4
Hyp (rel)	3.5 ± 0.5	3.3 ± 0.3
Hyp (abs)	3.4 ± 0.4	3.2 ± 0.3

Table 5.4 demonstrates the mean workload at peak exercise for both conditions. There was a main effect for condition (*normoxic vs. hypoxic, $P < 0.05$*), however, no interaction effect was observed (*condition x group, $P > 0.05$*). The inability to increase workload during maximal exercise in hypoxia may in part be due to a decrease in systemic O_2 concentration.

Table 5.5 –Peak heart rate ($b.min^{-1}$) scores in normoxia and hypoxia

Condition	*Normoxia*	*Hypoxia*
Norm	192 ± 7	192 + 7
Hyp (rel)	198 ± 11	192 + 10
Hyp (abs)	194 ± 6	194 + 6

Table 5.5 demonstrates the mean heart rate at peak exercise for both conditions. No between or within group differences were observed for heart rate at the point of exercise exhaustion.

Table 5.6 –Peak blood lactate (mmol.L^{-1}) scores in normoxia and hypoxia

Condition	*Normoxia*	*Hypoxia*
Norm	6.4 ± 2	7 ± 2.5
Hyp (rel)	8.7 ± 2.2	8.2 ± 1.7
Hyp (abs)	7.6 ± 2.3	8 ± 1.6

Table 5.6 demonstrates whole blood lactate concentration at peak exercise for both conditions. No between or within group differences were observed for blood lactate at the point of exercise exhaustion. Since 9 mmol.L^{-1} of lactate was not achieved in any group and all subjects were subjected to an environmental stressor, $\dot{V}O_{2\,max}$ can not be confirmed, hence the author has adopted the term $\dot{V}O_{2peak}$ (Brooks *et al*, 1996).

Table 5.7 –Peak oxygen saturation (%) scores in normoxia and hypoxia

Condition	*Normoxia*	*Hypoxia*
Norm	97 ± 2.4	89 ± 4.8
Hyp (rel)	95 ± 3.1	88 ± 7.3
Hyp (abs)	97 ± 3.1	89 ± 4.6

Table 5.7 demonstrates the mean arterial O_2 saturation at peak exercise for both conditions. There was a main effect for condition (*normoxic vs. hypoxic, $P < 0.05$*), however, no interaction effect was observed (*condition x group*, $P > 0.05$). It can be seen that exhaustive exercise in hypoxia decreases arterial O_2 saturation.

(C) Experimental physiological data

Figure 5.0 – Effect of acute hypoxia on oxygen uptake ($\dot{V}O_2$)

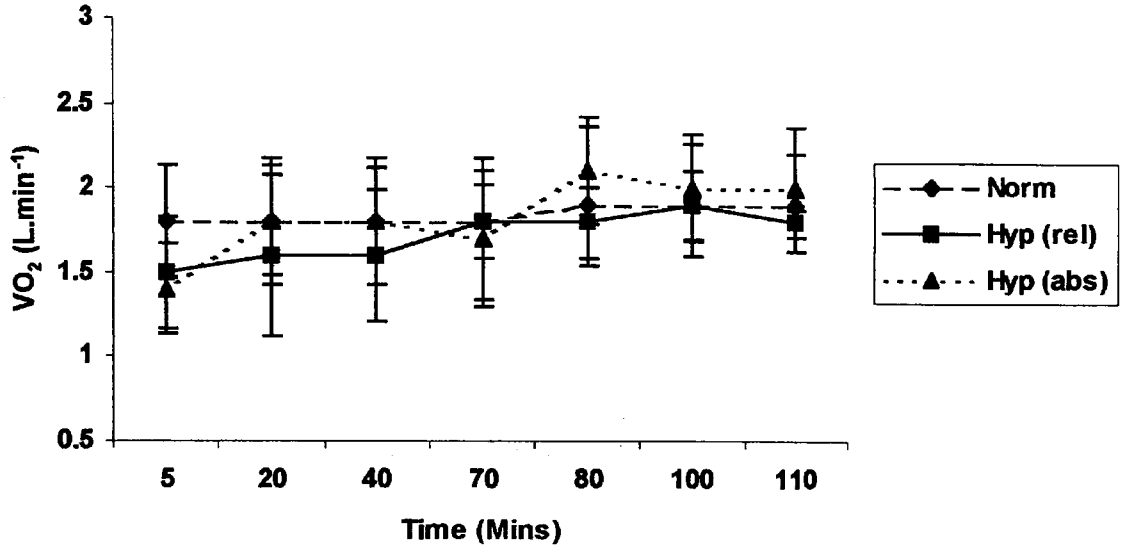


Figure 5.0 demonstrates the effect of acute exercise on oxygen uptake in normoxia and hypoxia. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), and an interaction effect for *group x time*. The following data (mins) represent within group effects ($P < 0.05$): *Norm group 5 vs. 70, 40 vs. 70; Hyp (rel) 5 vs. 70, 5 vs. 80, 5 vs. 100, 5 vs. 110, 40 vs. 100; Hyp (abs) 5 vs. 20, 5 vs. 40, 5 vs. 70, 5 vs. 80, 5 vs. 100, 5 vs. 110, 20, vs. 80, 40 vs. 80, 70 vs. 80, 70 vs. 100, 70 vs. 110, 80 vs. 110.* This data would suggest that aerobic exercise of this type increases whole body oxygen uptake.

Figure 5.1 – Effect of acute hypoxia on arterial oxygen saturation (SaO_2)

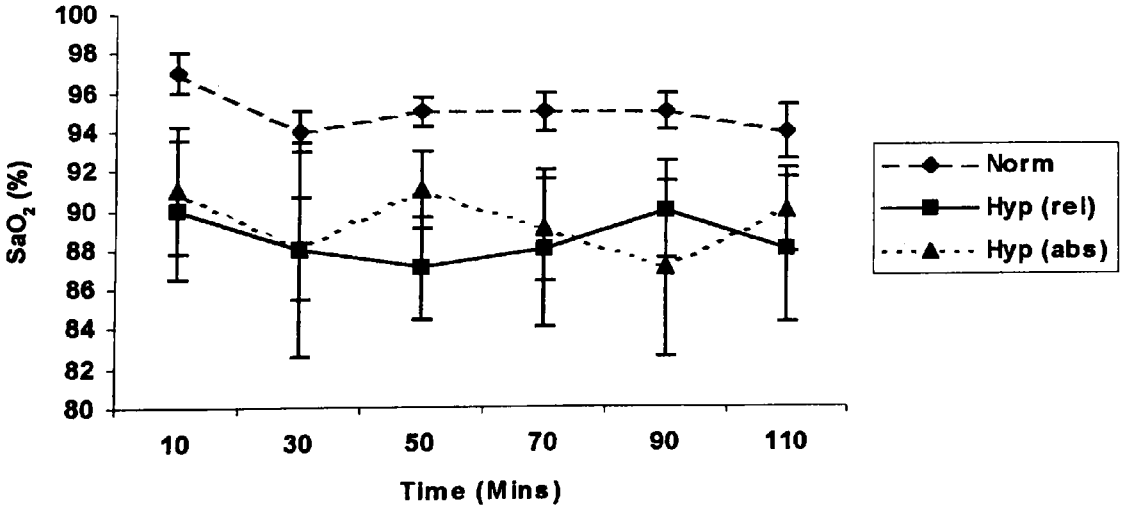


Figure 5.1 demonstrates the effect of acute exercise on arterial O_2 saturation in normoxia and hypoxia. There was a main effect for group (*norm vs. hyp (rel) and hyp (abs)*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$). From the data shown it is apparent that sustained exercise in hypoxia decreases arterial O_2 saturation; this may have profound implications for an exercise-induced oxidative stress as discussed later.

Figure 5.2 – Effect of acute hypoxia on heart rate

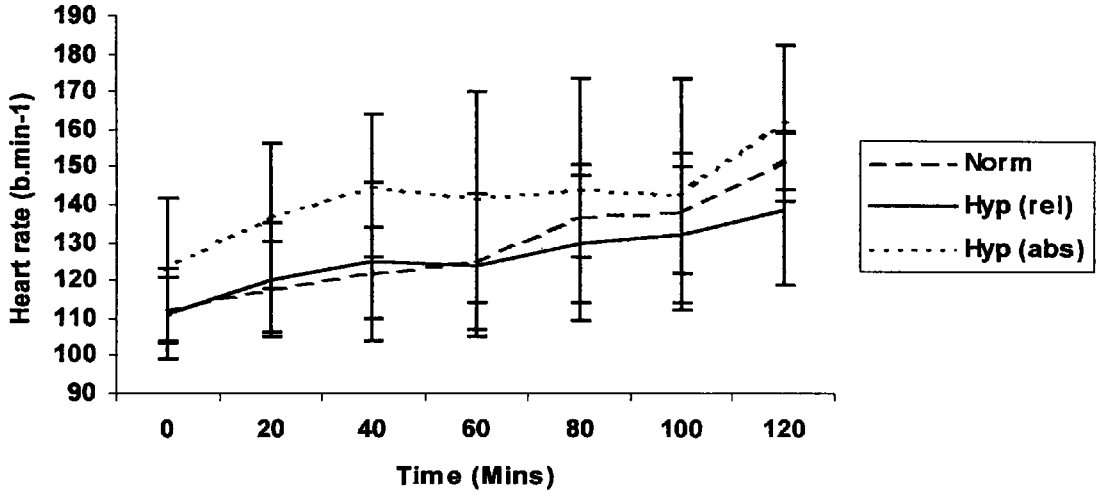


Figure 5.2 demonstrates the effect of acute exercise on heart rate in normoxia and hypoxia. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$). Physical exercise *per se* is shown to increase heart rate by 32% from rest to immediately post-exercise.

Figure 5.3 – Effect of acute hypoxia on rate of perceived exertion (RPE)

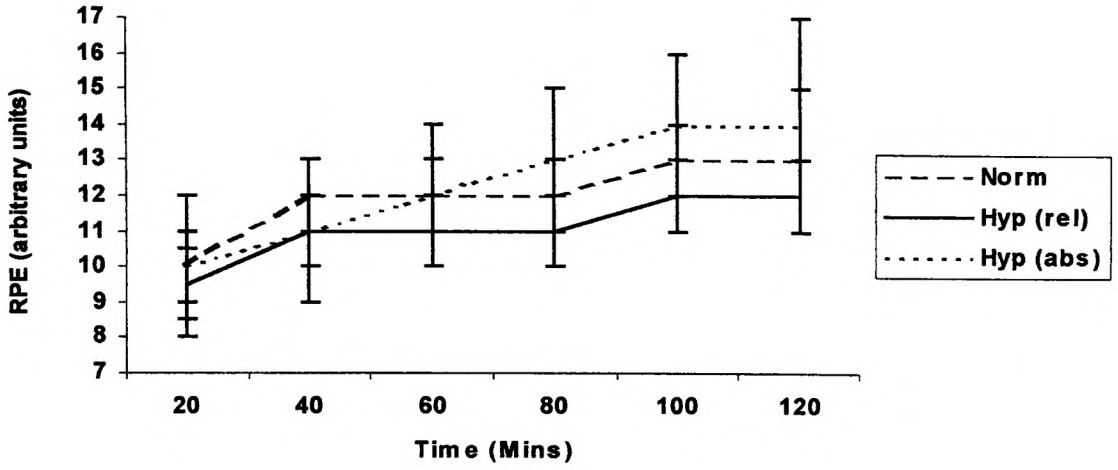


Figure 5.3 demonstrates the effect of acute exercise on RPE in normoxia and hypoxia. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$). Physical exercise *per se* is shown to increase RPE over time.

(D) Oxidative stress parameters

Figure 5.4 – Effect of acute hypoxia on PBN adduct concentration

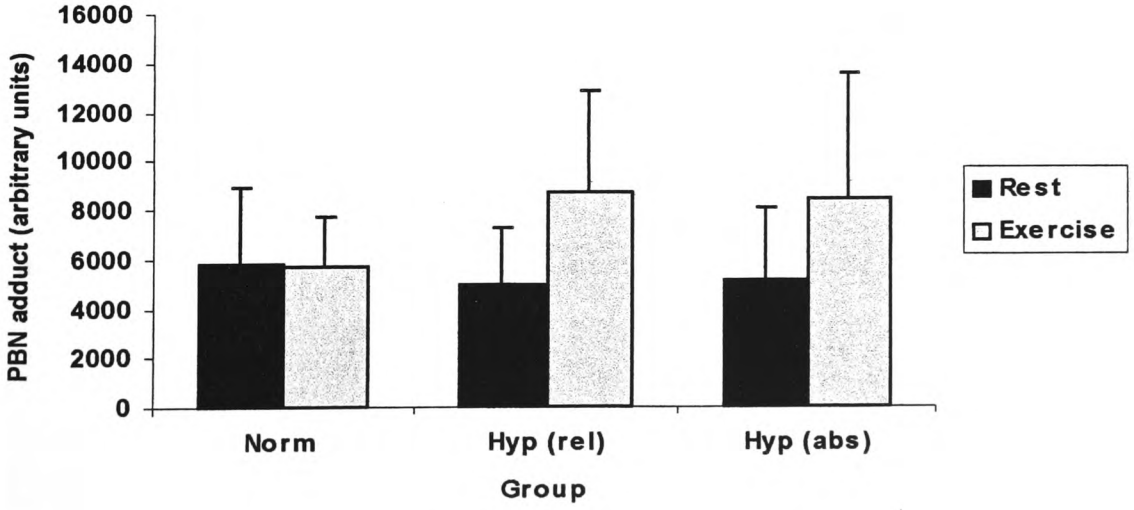


Figure 5.4 shows the effect of acute exercise on free radical generation in normoxia and hypoxia. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no

interaction effect was observed (*time x group,* $P > 0.05$; retrospective power calculation = 0.392). It may be observed that aerobic exercise increased the concentration of free radical species (*by 40.9%*) in human blood.

Figure 5.5 – Effect of acute hypoxia on lipid hydroperoxide (LH) concentration

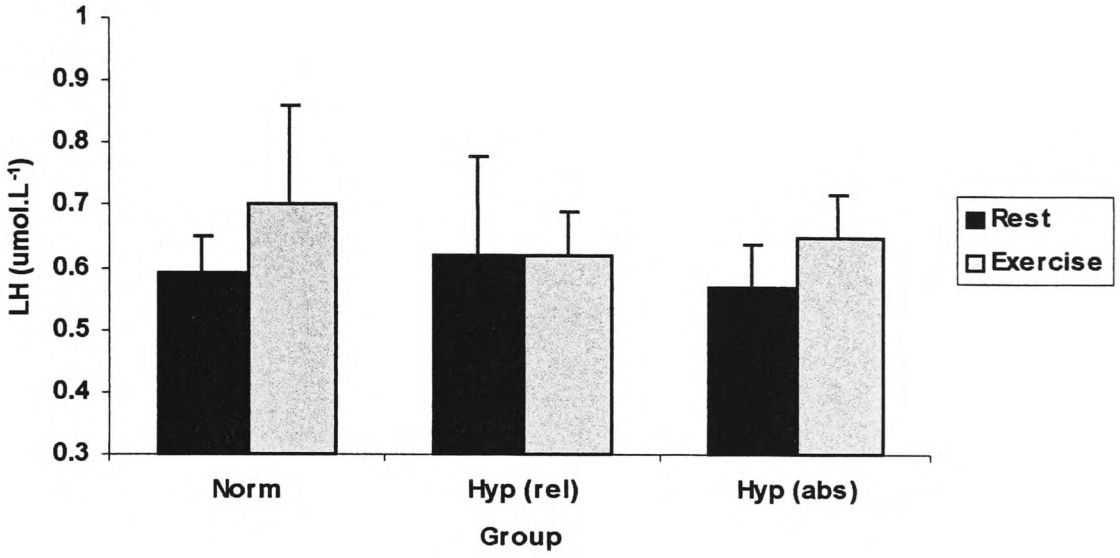
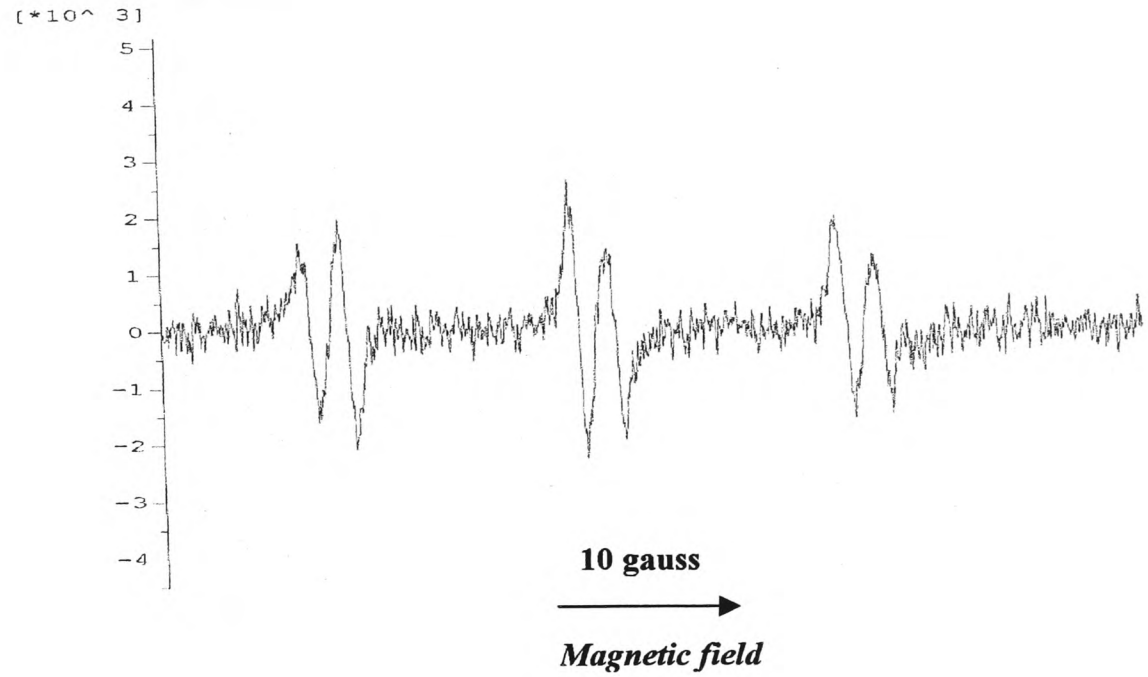


Figure 5.5 shows the effect of acute exercise on LH production in normoxic and hypoxic. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no interaction effect was observed (*time x group,* $P > 0.05$). This data implies that aerobic cycling exercise of this nature has the potential to increase venous LH concentration (*by 10.6%*).

Figure 5.6 – Typical rest (A) and (hypoxic) exercise (B) Electron Spin Resonance (ESR) spectra of α-phenyl-tert-butylnitrone (PBN) adducts in serum

(A) - Rest



(B) – Exercise

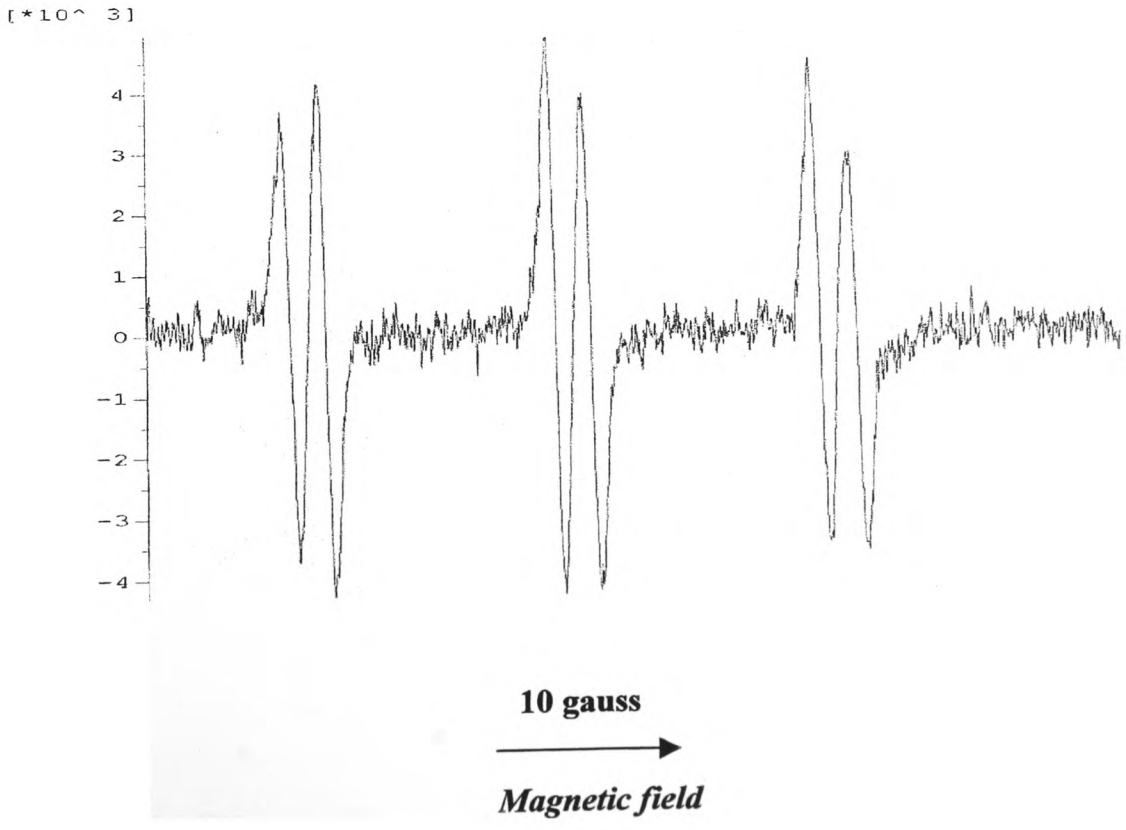


Figure 5.6 shows typical ESR spectra of the PBN adduct before and after aerobic exercise. For all ESR spectra of the PBN adduct detected *ex vivo* in human sera, the hyperfine coupling constants were measured at a_N = 13.8 gauss and $a\beta_H$ = 1.9 gauss. Therefore, the free radicals are cautiously identified as being secondary oxygen or carbon-centred lipid radicals, possibly derived from lipid hydroperoxide decomposition.

Figure 5.7 – Effect of acute hypoxia on malondialdehyde (MDA) concentration

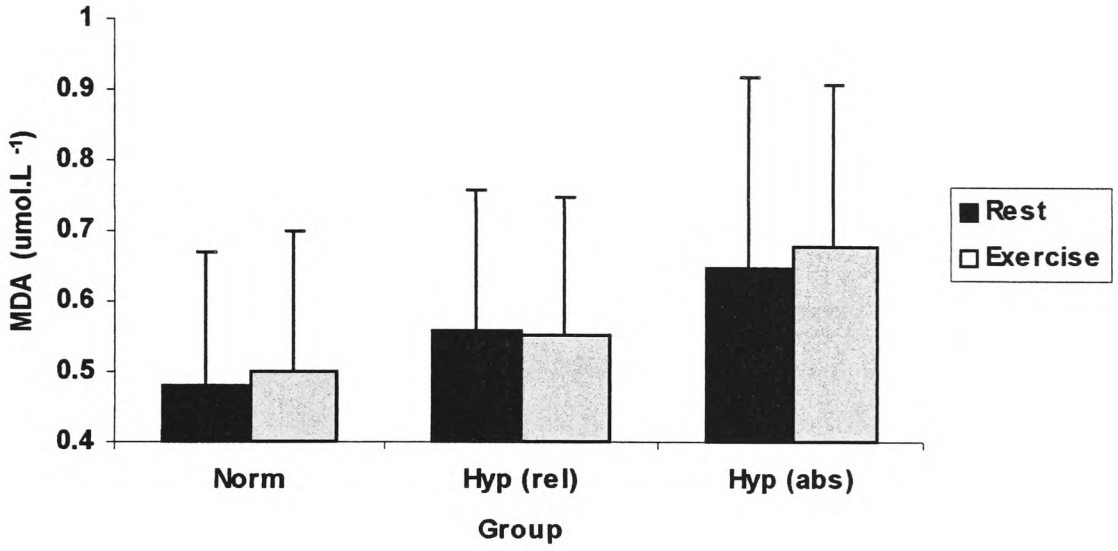


Figure 5.7 shows the effect of acute exercise on MDA production in normoxia and hypoxia. In contrast to the data outlined in figure 5.5, no between or within group differences were observed for MDA concentration during the low intensity exercise challenge.

Figure 5.8 – Effect of acute hypoxia on leukocyte concentration

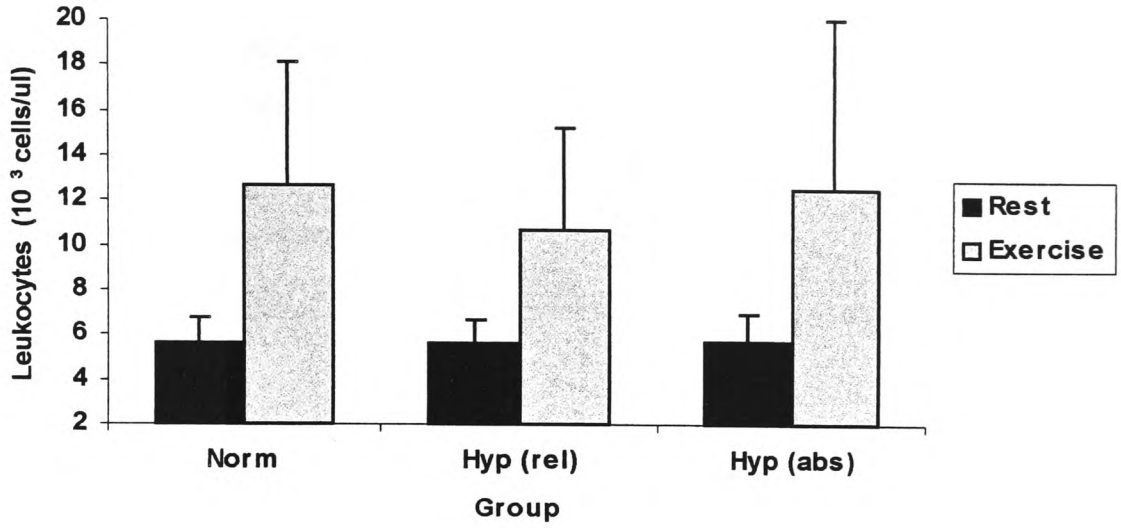


Figure 5.8 shows the effect of acute exercise on leukocyte production in normoxia and hypoxia. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$). Sustained cycling exercise increased leukocyte production, which incidentally may be involved in the primary free radical attack on cellular membranes.

Figure 5.9 – Effect of acute hypoxia on neutrophil concentration

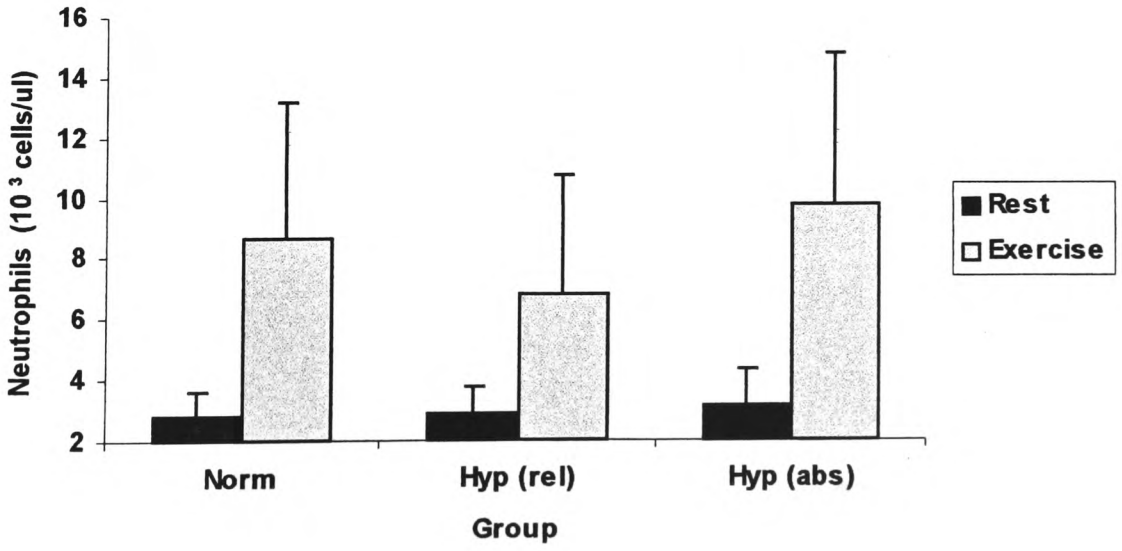


Figure 5.9 shows the effect of acute exercise on neutrophil production in normoxia and hypoxia. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$). Sustained cycling exercise increased neutrophil production, which may also be involved in the primary free radical attack on cellular membranes.

(D) Muscle damage parameters

Figure 5.10 – Effect of acute hypoxia on total creatine phosphokinase (CPK) concentration

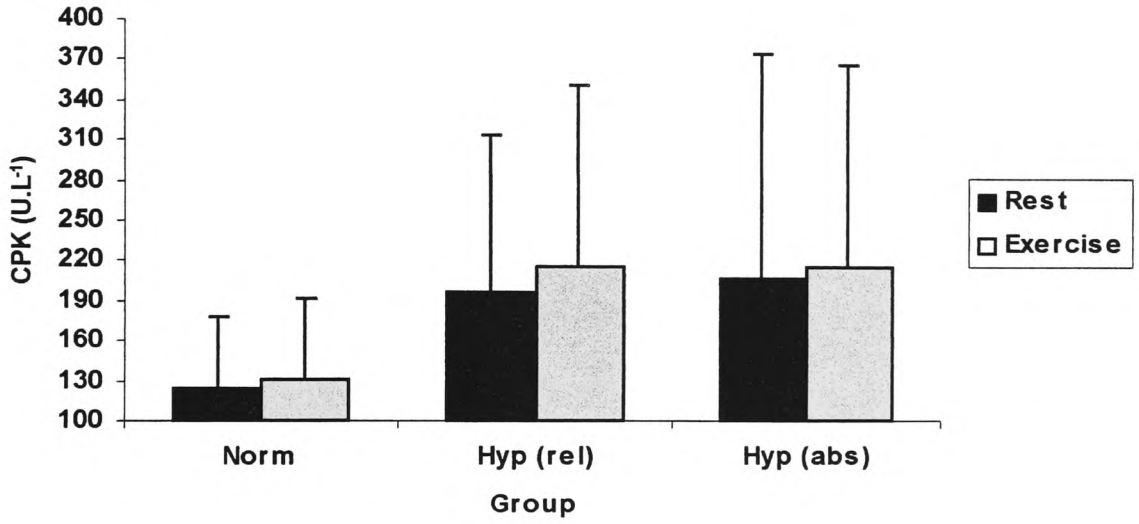


Figure 5.10 shows the effect of acute exercise on CPK production in normoxia and hypoxia. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$). This would suggest that sustained low intensity aerobic exercise causes muscle damage.

Figure 5.11 – Effect of acute hypoxia on myoglobin concentration

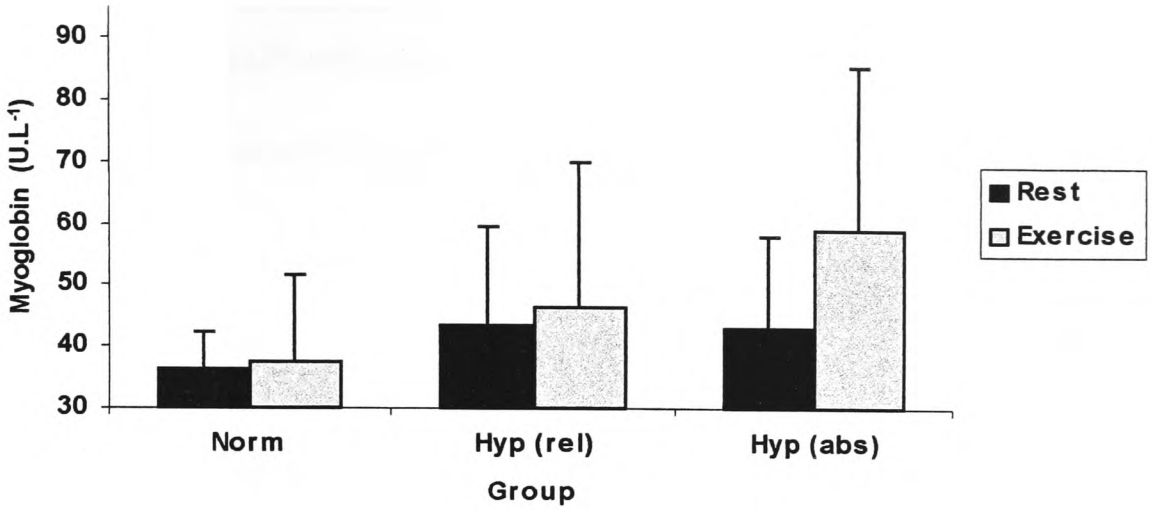


Figure 5.11 shows the effect of acute exercise on myoglobin production in normoxia and hypoxia. In contrast to the above observation, no between or within group differences were observed for myoglobin concentration.

(E) Antioxidant activity

Figure 5.12 – Effect of acute hypoxia on magnesium concentration

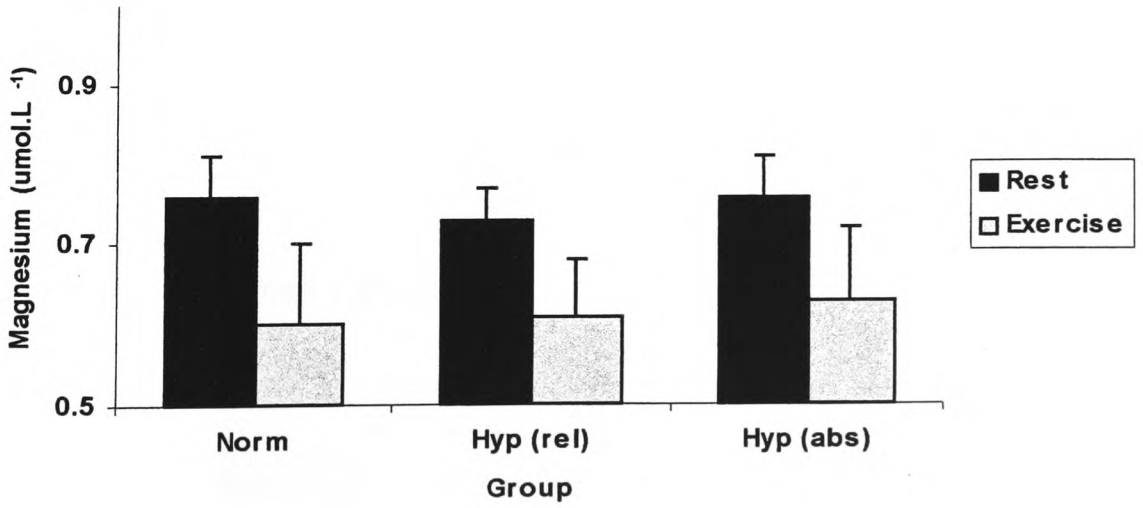


Figure 5.12 shows the effect of acute exercise on magnesium production in normoxia and hypoxia. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$). It can be seen that this type of aerobic exercise significantly decreases magnesium production.

Table 5.8 – Effect of acute hypoxia on selected antioxidant indices

Time	***Rest***	***Exercise***
Ascorbic acid		
Normoxic	60.8 ± 10.5	60.9 ± 16.4
Hypoxic (*rel*)	62.2 ± 10.4	61.5 ± 8.6
Hypoxic (*abs*)	51 ± 18.7	61.6 ± 1.7*
Interaction effect for time x group		***(P = 0.06)***
α-tocopherol		
Normoxic	19.7 ± 2.2	19.9 ± 4.3
Hypoxic (*rel*)	17.7 ± 3	19.1 ± 3.2
Hypoxic (*abs*)	18.2 ± 1.6	18.8 ± 2.7
NS		
α-carotene		
Normoxic	0.02 ± 0.008	0.02 ± 0.007
Hypoxic (*rel*)	0.01 ± 0.01	0.02 ± 0.01
Hypoxic (*abs*)	0.02 ± 0.01	0.02 ± 0.01
NS		
β-carotene		
Normoxic	0.06 ± 0.04	0.09 ± 0.08
Hypoxic (*rel*)	0.04 ± 0.02	0.05 ± 0.02
Hypoxic (*abs*)	0.07 ± 0.05	0.07 ± 0.05
NS		
Lycopene		
Normoxic	0.32 ± 0.08	0.27 ± 0.06†
Hypoxic (*rel*)	0.27 ± 0.10	0.30 ± 0.19
Hypoxic (*abs*)	0.35 ± 0.12	0.37 ± 0.13
Interaction effect for time x group		
Retinol		
Normoxic	0.74 ± 0.11	0.73 ± 0.22
Hypoxic (*rel*)	0.80 ± 0.15	0.80 ± 0.15
Hypoxic (*abs*)	0.76 ± 0.14	0.73 ± 0.15
NS		

Values are means ± SD, and expressed as $\mu mol \cdot L^{-1}$; n = 10 per group. NS, not significant; interaction effect (*time x group*) indicates a difference ($P < 0.05$) within

group as a function of time; † within group difference ($P < 0.05$); * within group difference ($P > 0.05$).

Table 5.8 demonstrates that aerobic exercise selectively decreased ($P < 0.05$) the venous concentration of lycopene by 18.5% in the normoxic group only. Although an interaction effect was detected for ascorbic acid, the post hoc test identified no significant differences. Therefore, it is accepted that aerobic exercise did not modify ascorbic acid concentration. No between or within group differences were observed for any other antioxidant metabolite.

Experimental inter-assay correlations

Please see methodology section 3.12.3 for explanation of correlations.

In the present investigation, a positive correlation was observed between the Δ (*exercise minus rest*) PBN adduct and $\Delta\dot{V}O_2$ ($r = 0.48$, $P < 0.05$). This association would suggest that increased oxygen flux may be implicated in the generation of free radical species.

No correlation was evident between Δ LH, Δ PBN or Δ CPK, nor was there any relationship between Δ LH, Δ CPK or Δ $\dot{V}O_2$. An extremely weak inverse association was apparent between Δ LH and Δ vitamin E ($r = -0.22$), however this relationship was non-significant ($P > 0.05$) and may purely be due to chance.

A strong association was observed between Δ neutrophil and Δ leukocyte production ($r = 0.85$, $P < 0.05$).

5.3 – DISCUSSION

The present investigation aimed to measure and quantify oxidative stress in acute normobaric hypoxic and normoxic aerobic exercise. The main finding of this study reports that aerobic exercise but not hypoxia, markedly increased the concentration of free radical species and lipid hydroperoxides (LH) in systemic blood. This increase in

exercise-induced oxidative stress was paradoxically related to an increase in oxygen consumption and a selective decrease in arterial O_2 saturation, implicating the mitochondria as a potential source of reactive oxygen species (ROS) (Boveris and Chance, 1973). Aerobic exercise activated circulating leukocyte and neutrophil activity, which may have also stimulated primary oxygen radical production. The increase in creatine phosphokinase (CPK) activity during exercise would suggest increased sarcolemmal permeability, and this may be caused by free radical attack to the sarcolemmal membrane (Hikida *et al*, 1983). Little evidence was apparent of an activated antioxidant defence system, postulating that exercise of this intensity and duration may overwhelm antioxidant activity to cause oxidative damage.

Aerobic exercise and oxidative stress

Direct (*ESR*) evidence of free radical generation during physical exercise is limited to a few investigative reports in the human (Davison *et al* 2001, Ashton *et al* 1998) and animal model (Davies *et al* 1982, Jackson *et al* 1985). Recent indirect (*by-products of oxidation*) evidence suggests that physical exercise performed in hypoxia may stimulate free radical production and oxidative injury (Bailey *et al* 2000, Bailey *et al* 2001), however this hypothesis has not been confirmed directly in moderate aerobic exercise in hypoxic conditions.

Direct evidence using ESR shows that moderate aerobic exercise in hypoxia does not increase free radical concentration. This finding is at variance with previous reports within the literature. Other studies however in hypoxia have largely used short-term exhaustive protocols (*i.e.* at or near $\dot{V}O_{2\,max}$) to generate an oxidative stress (Bailey *et al* 2000 and 2001), whilst the exercise intensity was much lower in the present study. Nevertheless pooled data would suggest that 2 hours of exercise at 55% $\dot{V}O_{2peak}$ generates free radical species within the systemic circulation to levels beyond the capacity of the antioxidant defence system.

The hyperfine coupling constants of all *ex vivo* PBN trapped free radicals were consistently the same, and are suggestive of secondary oxygen-centred alkoxyl radicals. Ashton *et al* (1998) were the first scientists to report the hyperfine coupling constants (a_N = 13.7 gauss, $a\beta_H$ = 1.9 gauss) from strenuous exercise in human blood, and they

compare favourably to those observed in the present investigation. The authors suggest that the species detected are secondary alkoxyl free radicals, thus supporting conclusions drawn in the present study. Other supporting evidence of similar coupling constants and radical identification may be obtained from the work of Tortolani *et al* (1993), Bolli *et al* (1988), Coghlan *et al* (1991) and Grech *et al* (1996).

Coupled with the fact that lipid hydroperoxide concentration increased as a result of moderate exercise, the author postulates that the primary source of the alkoxyl radical observed in the present study is via initial primary oxygen-centred radical attack and subsequent PUFA decomposition (Duthie, 1993). Lipid peroxidation is a self-perpetuating chain reaction, which generates oxidation by-products and free radical intermediates (Ashton *et al*, 1998). Furthermore, it has been suggested that PBN adducts may be formed from analogues of lipid hydroperoxides (Mergner *et al*, 1991).

In support, Ashton *et al* (1998) speculate that the alkoxyl radical results from lipid peroxidation of cellular membranes by primary free radical attack resulting in increased levels of lipid hydroperoxides post-exercise. Tortolani *et al* (1993) also claim that the alkoxyl radicals observed from patients undergoing elective cardioplegia are formed as a result of lipid hydroperoxide breakdown following an iron dependent reaction. These findings have been confirmed by Coghlan *et al* (1991), who detected PBN trapped radicals in the presence of lipid hydroperoxides in blood samples from patients undergoing coronary angioplasty. Direct free radical detection, in addition to a rise in LH concentration in the present study, provides convincing evidence that the damage inflicted to cellular membranes during aerobic exercise is in fact free radical mediated.

There are many cells and organs in the body that posses a lipid bi-layer with double bond structures; which by definition are susceptible to primary oxygen radical attack. Therefore it would be virtually impossible to suggest an exact location for PUFA oxidation; hence the author suggests two potential sites of primary free radical generation and lipid peroxidation during aerobic exercise.

➢ *Mitochondria*

Moderate to intense physical exercise is known to increase systemic oxygen uptake by approximately 35 fold above resting levels (Aw *et al*, 1986) and up to 200 fold in active peripheral skeletal muscle fibres (Keul *et al*, 1972). The mitochondrial electron transport chain within a muscle fibre has long been considered the major site of ROS production at rest and during exercise (Di Meo and Venditti, 2001). Studies on isolated mitochondria suggest that between 2 to 5% of total electron flux through the cytochrome chain may undergo one electron univalent reduction, with the formation of superoxide and hydrogen peroxide (Boveris and Chance 1973, Loschen *et al* 1974, Cadenas *et al* 1977, Turrens and Boveris 1980). Therefore with a mass action effect of systemic $\dot{V}O_2$ evident during physical exercise, it has been suggested that increased electron flux through oxidative phosphorylation may increase ROS production (Sjödin *et al* 1990, Benzi 1993, Alessio 1993).

The exact site of ROS production within active muscle mitochondria has been the subject of much recent debate (Di Meo and Venditti, 2001). The potential site of single electron reduction was identified in studies using animal heart mitochondria (Takeshige and Minakami 1979, Loschen *et al* 1971, Turrens and Boveris 1980), and implicates the NADH coenzyme Q reductase (Complex I) and ubiquinone-cytochrome bc_1 (Complex III) of the electron transport chain. Unfortunately, the mitochondrial complexes are located near the outer mitochondrial membrane, which can facilitate the rapid diffusion of electrons and subsequent binding with oxygen molecules to form superoxide radicals. It is pertinent to point out that presenting the mitochondria with an increased flux of oxygen, for example during exercise, does not result in increased free radical formation, but an increase in electron leakage from the respiratory chain which as described can produce increased amounts of superoxide radicals (Gutteridge and Halliwell, 1994).

Given the positive association between $\dot{V}O_2$ and the PBN adduct and the significant rise in oxygen uptake from rest to post exercise in the present study, it is postulated that aerobic exercise increased tissue oxygen flux, causing an increase in electron pressure within the mitochondrial respiratory chain with rapid formation of primary oxygen-centred radical species (*e.g.* $O_2^{\cdot-}$) (Sjödin *et al* 1990).

In support, endurance exercise has been shown to produce ESR detected semiquinone radicals, derived from inner mitochondrial membrane "*leakiness*" (Davies *et al*, 1982). Confirmation of the species was obtained from other skeletal muscle homogenates during electrical stimulation (Jackson *et al*, 1985). More recent evidence using a microdialysis probe have demonstrated a rise in the reduction of cytochrome *c*, suggesting an increase in superoxide production within the interstitial space of contracting mouse muscle (McArdle *et al*, 2001). These data provide conclusive evidence that free radicals are formed in exercising skeletal tissue, and supports the hypothesis that mitochondria *per se* may induce the formation of free radical species.

Indirect support of a greater blood flow to skeletal muscle, hence increased O_2 consumption within muscle mitochondrial fibres, may be obtained from the increase in heart rate from rest to exercise. As cardiac output is a product of heart rate (Brooks *et al*, 1996), it can be assumed that oxygen delivery to skeletal tissue is enhanced, thus providing a greater O_2 flux within the respiratory chain, yielding increased amounts of ROS. Furthermore, heart rate is known to increase in proportion to oxygen uptake (Saltin *et al*, 2000). Other data from Jenkins *et al* (1984) showing a positive correlation between $\dot{V}O_{2\,max}$ and the level of superoxide dismutase and catalase in vastus lateralis muscle, provides further support for the proposed mitochondrial source of primary oxygen radicals.

However once superoxide is generated within the mitochondria, it is important to consider the limited $O_2^{\cdot-}$ diffusibility across the mitochondrial and outer cellular membrane (Halliwell and Gutteridge, 1999), and the presence of manganese superoxide dismutase (MnSOD) in the dismutation reaction of $O_2^{\cdot-}$ to hydrogen peroxide (H_2O_2) (McCord and Fridovich, 1969). The author suggests that H_2O_2 is perhaps a more important and dangerous reactive oxygen species (ROS) due to its ability to diffuse more readily across membranes into extracellular compartments (Halliwell and Gutteridge, 1999), and aid in the generation of other highly toxic free radical species that have the potential to initiate chain propagation (*e.g.* hydroxyl radical) (Duthie, 1993).

Therefore, it is implied that the observed increase in PBN adduct concentration after exercise is due to the primary radical species attacking either intracellular or

extracellular polyunsaturated fatty acids, which would decompose lipid hydroperoxides in the present of iron, yielding a rapid rise in alkoxyl radical formation, allowing subsequent detection via ESR spectroscopy. This theory is supported by the increase in lipid hydroperoxide concentration post-exercise and confirms the origin of the PBN adduct. Since the inner and outer mitochondrial membrane are made up of approximately 20% and 50% lipid (Devlin, 1997), there is no doubt that lipid hydroperoxides may be formed within the mitochondria and subsequently diffuse into the systemic circulation. Evidence is provided for the presence of free iron to aid in such a reaction by Jenkins *et al* (1993), who has demonstrated that iron becomes more loosely bound to transferrin during physical exercise. Moreover, the membrane may provide reducing power, for example, sulphydral groups on membrane proteins may reduce Cu^{2+} to Cu^{+} (Minotti and Aust, 1987), and the microsomal electron transport chain has been shown to reduce Fe^{3+} to Fe^{2+-} if NADPH is supplied (Aust *et al*, 1985).

Central to the above hypothesis is that the antioxidant activity within skeletal muscle is more than one order of magnitude lower than of other highly aerobic tissues such as liver (Di Meo and Venditti, 2001), therefore should ROS formation increase in proportion to mitochondrial O_2 flux, the sarcolemma would undergo massive oxidative damage during exercise (Di Meo and Venditti, 2001). This is supported not only by the increase in lipid hydroperoxide and CPK concentration in the present study, but also by data showing a loss of sarcoplasmic and endoplasmic reticulum integrity in exercising animals (Davies *et al*, 1982).

As stated previously, lipid hydroperoxide concentration increased after exercise, and is the first conclusive evidence of an increase in lipid hydroperoxides after 2 hours of aerobic exercise at 55% $\dot{V}O_{2peak}$. This data is in general agreement with the work of Ashton *et al* (1998), who has demonstrated an increase in lipid hydroperoxide following acute maximal aerobic exercise. More importantly, Ashton *et al* (1998) showed an increase in ESR signal intensity, confirming that the free radicals detected are in fact lipid-derived, originating possibly from lipid hydroperoxide decomposition. Furthermore, decreased intensity in the high field lines of the ESR spectrum (*figure 5.6*) usually indicates spin-inhibition, which may be the result of a large molecule such as a long chain polyunsaturated fatty acid of membrane origin attached to the PBN spin trap molecule (Ashton, 1998).

Since evidence of membrane peroxidation is provided, it may be suggested that the integrity of the cellular membrane is compromised particularly as an increased release of the glycolytic protein creatine phosphokinase (CPK) was evident. The presence of systemic CPK provides evidence for sarcolemmal permeability (Child, 1997), which during prolonged exercise could arise from sarcolemmal membrane rupture (Hikida *et al* 1983, Child 1997), possibly as a result of free radicals produced during exercise (Ebbeling and Clarkson, 1989). In providing support, Child *et al* (1998) suggest that as exercise duration increases, peroxidation damage may be more important than mechanical factors in elevating myocellular enzyme release. Kanter *et al* (1988) has shown a positive correlation between CPK and lipid peroxidation. Sjödin *et al* (1990) suggest that elevated lipid peroxidation levels and the appearance of muscle proteins within the plasma, may indicate an association between the initiation of exercise-induced free radical attack and the loss of mitochondrial membrane integrity resulting in the efflux of muscle derived enzymes. However, no relationship between oxidative stress and muscle damage proteins were observed in this study, which is in agreement with Child *et al* (1998) showing no correlation between lipid peroxidation and CPK after aerobic exercise.

CPK is a commonly used marker of muscle damage as between 90 to 100% is located in skeletal muscle tissue. However, several factors need highlighting regarding protein efflux, including protein transportation from the muscle interstitium to the intravascular space through the lymphatic vessels, and their clearance rate (Ebbeling and Clarkson 1989). The higher CPK levels observed following exercise were probably due to the greater free radical concentration and disruption of the muscle cell membrane and possible accelerated transport to the venous circulation.

The above data would support the theory that increased mitochondrial electron '*leakage*' is a viable source of primary oxygen free radicals that can peroxidise lipids and cause exercise-induced muscle damage. The selective increase in CPK and not myoglobin however warrants further investigation.

Although aerobic exercise in hypoxic *per se* did not selectively increase free radical generation, other work concerned with hypoxia have demonstrated an increase in lipid

peroxidation. The interpretation of these findings may have some relevance to the overall increase in oxidative stress in the present investigation.

Simon-Schnass and Pabst (1988) observed a significant increase in free radical mediated lipid peroxidation at 8611 m above sea level, and suggests that the hypoxic cells were particularly susceptible to oxidative stress, due to an accumulation of electrons that cannot be transferred to O_2 at the level of mitochondrial cytochrome oxidase due to a decline in cellular respiration. This would no doubt increase electron pressure and leakage, and in doing so increase superoxide formation. Notwithstanding the fact that overall O_2 consumption increased in the present study, there was a selective decrease in arterial haemoglobin O_2 saturation between both hypoxic and normoxic groups. This would suggest a decrease in diffusive O_2 delivery to active skeletal myocytes, causing a reduction in cytochrome oxidase turnover and an increase in univalent reduction.

More recent work by Bailey and co-workers (2001) have shown an increase in lipid hydroperoxide and malondialdehyde concentration following exhaustive exercise in normobaric hypoxia (F_IO_2 = 16%) despite a selective exercise-induced mobilisation of α-tocopherol. This increase in lipid peroxidation was associated with a decrease in arterial haemoglobin O_2 saturation (LH, r = -0.61, $P < 0.05$; MDA, r = -0.50, $P < 0.05$) as opposed to maximal O_2 consumption. These authors suggest that an exercise-induced mass action effect of mitochondrial O_2 flux is not the exclusive mediator of ROS production, and claim that a decrease in mitochondrial PO_2 ($P\textit{mit}O_2$) as opposed to the original mitochondrial O_2 flux concept, more precisely regulates mitochondrial and/or systemic ROS generation. In support of the theory devised by Bailey *et al*, recent *in vitro* evidence suggests that the mitochondria can detect and respond to a decline in intracellular PO_2 via a complex signal transduction process which involves an increase in ROS (Chandel and Schumacker, 2000). The ROS may act as second messengers in the cellular adaptive responses that ultimately served to defend $P\textit{mit}O_2$ and thus protect against cellular anoxia (Chandel and Schumacker 2000, Bailey *et al* 2001). In agreement with Bailey *et al* (2001), the hypoxic groups in this study presented with a selective decrease in arterial haemoglobin O_2 saturation, therefore perhaps this proposed mechanism partially contributed to the increase in free radicals and lipid hydroperoxide observed. To confirm whether a cause and effect association

exists between P*mit*O_2 and free radical generation, investigators are encouraged to combine the techniques of ESR and proton magnetic resonance spectroscopy (Bailey *et al* 2001, Bailey 2001).

Hypoxic cells are also known to cause ATP depletion which may cause the ATP-dependent Ca^{2+} pumps to malfunction (Sjödin *et al,* 1990). This occurrence may raise intracellular free Ca^{2+} and generate superoxide radicals as described in section 2.2.7. A rise in intracellular Ca^{2+} can also activate phospholipase A_2 causing cell membrane disruption, allowing the efflux of cytosolic enzymes into the intravascular space (Jackson, 1998). The increase in CPK detected may support this proposed mechanism.

It would be unlikely that xanthine oxidase is the source of ROS in the present study due to the fact that blood was obtained *during* the exercise protocol, thus if muscular ischaemia was experienced during exercise, tissue reoxygenation and subsequent ROS formation would not transpire until the cessation of exercise. This conclusion may also be apparent for other organs (*e.g.* kidney and stomach) that may also become partially ischaemic due to the redistribution of blood flow to active skeletal muscle fibres during exercise (Witt *et al*, 1992). This view is upheld by the work of Mori *et al* (1999) demonstrating a peak in hypoxanthine concentration 20 minutes after exercise at 50% of maximal work rate performed in a hypoxic chamber.

➢ *Systemic circulation*

The previous section adopted an intracellular approach as a viable mechanism for an increase in exercise-induced oxidative stress. However, the free radicals observed in the present study may have equally originated from an extracellular source. It is suggested that the aerobic exercise challenge may have activated white blood cells to produce primary oxygen radical species that have the capability to damage surrounding fatty acid molecules.

Activated vascular leukocyte and neutrophil cells are known to produce ROS (Santos-Silva *et al*, 2001) and both were increased following exercise and also highly correlated in the present study. As oxygen uptake increased (*causing bodily stress*) during exercise, it is postulated that phagocytosis activated membrane-bound NADPH oxidase

which reduced molecular oxygen to superoxide (Suzuki *et al* 1996, Gordon *et al* 2000). This free radical can then either peroxidise lipid membranes, up-regulating venous lipid hydroperoxide concentration or may be dismutated by superoxide dismutase in forming H_2O_2. H_2O_2 may either form the damaging $OH^{\cdot}$ radical by an iron catalysed reaction, or as the presence of neutrophilia would suggest, increase activation of the enzyme myloperoxidase in forming hypochlorous acid, a highly reactive molecule known to cause damage to biomolecules (Halliwell and Gutteridge, 1999). Whichever path H_2O_2 takes, it is inevitable that a more damaging ROS is produced which may target and damage lipid structures. As erythrocyte membranes are rich in PUFA and highly susceptible to oxidative damage (Clemens and Waller, 1987), it is likely that the increase in venous lipid hydroperoxide and lipid alkoxyl radicals originate from this source. Additionally, as lipid radicals in particular are known to travel within the blood, they may propagate and attack PUFA other than their site of formation (Sen and Hanninen, 1994), causing mass destruction within the body and at the same time allowing more free radicals to be generated and detected.

In support of the proposed mechanism, recent evidence has shown that exercise stimulates leukocyte activation causing an increase in red blood cell damage and lipoperoxidation (Santos-Silva *et al*, 2001). These authors conclude that the findings warrant a re-evaluation of current views in the intensity, duration and regularity of physical exercise, and that the evaluation of leukocyte activation products, erythrocyte damage and oxidative stress may represent good markers to establish protective thresholds.

In providing further support to the proposed extracellular source of ROS, it has been demonstrated that endurance training can (1) elevate antioxidant enzyme activity (SOD and GPX) in erythrocytes, and (2) decrease neutrophil $O_2^{\cdot-}$ production in response to exhausting aerobic exercise. Moreover, this up-regulation in antioxidant defence was accompanied by a reduction in exercise-induced lipid peroxidation in erythrocyte membrane (Miyazaki *et al*, 2001). Other ESR data have isolated the presence of the $OH^{\cdot}$ radical in inflamed human phagocytes (Cohen *et al*, 1991), providing evidence of other free radical involvement in this mechanism as opposed to $O_2^{\cdot-}$ only.

Aerobic exercise and antioxidant activity

Although aerobic exercise had little obvious effect on the majority of plasma antioxidants, there was some antioxidant activity as demonstrated by the selective decrease in lycopene and magnesium post-exercise. It is hypothesised that these antioxidants responded to the systemic increase in alkoxyl radical generation as evident by the lack of MDA concentration.

Lycopene is known to have general antioxidant qualities with possible free radical scavenging ability (Halliwell and Gutteridge, 1999), however little is currently known with regard to the antioxidant properties of magnesium. Magnesium is a cofactor for multiple enzymes *in vivo* (*i.e.* pentose phosphate pathway) (Halliwell and Gutteridge, 1999), and is known to increase oxidative stress in a deficient state (Kharb and Singh, 2000). As magnesium decreased during exercise to a concentration half the resting value, it is speculated that it contributed to the scavenging of the alkoxyl radical. Thus the detected concentration in PBN adduct observed may seriously underestimate the absolute free radical production during physical exercise.

In support of the antioxidant capabilities of magnesium, Garcia *et al* (1998) used ESR spectroscopy to conclude that magnesium can attenuate the increase in free radical species after a brief coronary occlusion-reperfusion sequence. Similarly, Zhou *et al* (1999) demonstrated that magnesium deficiency can increase H_2O_2 production in cultured human endothelial cells. Although research would suggest that magnesium has some antioxidant potential, more research is warranted to determine the antioxidant properties of magnesium.

It is proposed that responses of both magnesium and lycopene were insufficient to prevent exercise-induced lipid peroxidation, indicating an imbalance in the antioxidant defence network at the sites of oxygen radical formation during aerobic exercise.

5.4 - CONCLUSION

Exercise has always been regarded as an important component of human longevity, due to its ability to decrease the factors (*e.g.* LDL cholesterol) that contribute towards

cardiovascular related disease (Bouchard, 1994). However, Jenkins (2000) recently asked an important question in what type of physical exercise could increase oxidative stress, and may this exert potentially adverse effects on human health? This study has measured and quantified oxidative stress in acute aerobic exercise, and demonstrates for the first time that prolonged aerobic exercise performed at 55% of $\dot{V}O_{2peak}$ can increase free radical activity, as measured by ESR spectroscopy. Exercise performed in hypoxia did not increase any oxidative stress parameter, which is related perhaps to exercise intensity.

In addition to an exercise-induced increase in the PBN adduct, exercise of this duration and intensity also increased lipid hydroperoxide and CPK production, which clearly suggests cell membrane damage, and increased sarcolemmal permeability. These findings challenge the current thoughts of Vina *et al* (2000), who state that changes in oxidative stress and muscle damage biomarkers do not depend on the absolute intensity of exercise but occur only when exercise is performed to exhaustion.

It is apparent that exercise performed for 2 hours at 55% of $\dot{V}O_{2peak}$ increases free radical production and lipid peroxidation, however it is extremely difficult to ascertain whether or not the appearance of these metabolites in the peripheral circulation provides sufficient evidence to suggest that exercise of this nature may cause significant perturbations to human health. In hindsight, the measurement of oxidised DNA and LDL cholesterol may provide evidence for the potentially hazardous effects of aerobic exercise. However, this author indirectly claims that exercise of this type is *not* detrimental to human health as the percentage change from rest to exercise for both oxidative stress parameters were within the pre-determined critical difference levels. The free radical concentration changed by 40.9% and the lipid hydroperoxide concentration by 10.6%, well within their respective critical difference levels of 121% and 27.5%. Therefore, although statistical significance was evident in the present study, exercise of this type failed to cause any *real* physiological change, thus the claim that aerobic exercise performed at 55% of $\dot{V}O_{2peak}$ increased oxidative stress must be viewed with caution.

Additionally, Jenkins and Goldfarb (1993) postulates that although oxidant stress may occur during exercise, it is unlikely that such stress may cause substantial damage to healthy individuals since the body is saturated with an array of protective substances. Results of this study however suggest that aerobic exercise did not alter endogenous antioxidant activity, claiming that exercise of this nature increased electron flux through the rapidly respiring mitochondria and/or activated circulating leukocytes without causing significant antioxidant adaptation in order to cope with the increase in free radical species. The latter claim is weakened however by the fact that endogenous antioxidant status in the present investigation was confined to the systemic circulation, while the measurement of intracellular compounds may have proven useful.

Chapter 6

Study 2

Exercise and Oxidative Stress in Type 1 Diabetes Mellitus

6.0 – INTRODUCTION

Free radicals have the potential to cause damage to critical cellular targets, such as lipid membranes as a consequence of increased mitochondrial oxygen (O_2) flux (Alessio, 1993). This notion was formulated as a result of studies conducted by Dillard *et al* (1978) and Davies *et al* (1982), where physical exercise, elevated O_2 consumption, and oxidative stress was related in humans and rats respectively. Assuming a 200 fold increase in O_2 flux within skeletal muscle during exhaustive physical exercise (Keul *et al*, 1972), it is conceivable that an increase in metabolism can overwhelm endogenous antioxidant defences to form free radical species. Apart from the cellular damaged inflicted by free radicals induced by physical exercise, oxidative stress has been implicated in a wide variety of pathologies, including cancer, atherosclerosis, hypertension, cataracts and liver disease (Alessio, 1994). Recent reports suggest that patients with diabetes mellitus are susceptible to increased levels of oxidative stress (Santini *et al* 1997, Griesmacher *et al* 1995). However, controversy exists over whether the increased reactive oxygen species (ROS) are merely associative rather than causal (Laaksonen and Sen, 2000). Poor metabolic control may facilitate an increase of superoxide anions ($O_2^{\cdot}$) in diabetic serum (Ceriello *et al,* 1991). Other potential mechanisms relating to enhanced oxidative stress in diabetes point to compromised antioxidant defences, glucose auto-oxidation, formation of advanced glycated end products and a change in the glutathione redox status (Laaksonen and Sen 2000, Giugliano *et al* 1996). Recent Electron Spin Resonance (ESR) spectroscopic evidence has shown a post-prandial increase in the concentration of free radicals in patients with type 2 diabetes (Anderson *et al,* 2001), presumably due to increased substrate availability and/or increased metabolism due to the thermic effect of food. At present, there is a paucity of data on the effects of exercise in type 1 diabetes in relation to free radical production. Only two studies have examined oxidative stress in relation to acute exercise in type 1 diabetes. Laaksonen *et al* (1996) and Atalay *et al* (1997), have shown that patients with type 1 diabetes mellitus are exposed to increased basal as well as exercise-induced lipid peroxidation, while paradoxically, exercise is recommended in the management of patients with type 1 diabetes (American Diabetes Association, 1993). A limitation of these studies is that only indirect assays are used. ESR spectroscopy is the most direct method available of detecting free radical species in human blood (Holley and Cheeseman, 1993), and until recently was largely confined to

the animal model (Davies *et al* 1982, Jackson *et al* 1985). However, using the spin trap technique, several human studies have utilised ESR spectroscopy to examine oxidants in several pathological cases including angioplasty (Grech *et al,* 1996), cardioplegia (Tortolani *et al,* 1993), and type 2 diabetes (Anderson *et al* 2001, Delmas-beauvieux *et al* 1998). There are currently no published studies that have used ESR spectroscopy with or without exercise to examine free radical concentration in type 1 diabetes. Therefore the present study was designed to quantify free radicals directly using ESR and the spin trap α-phenyl-*tert*-butylnitrone (PBN), together with supporting assays of lipid peroxidation.

6.1 - METHODOLOGY

Subject characteristics

Twelve (n = 12) male type 1 diabetic patients (diabetic group) and thirteen (n =13) apparently healthy male volunteers (control group) were recruited for the present study (*table 6.0 for subject characteristics*). Diabetic patients were microalbuminuria negative and had no known diabetic complications. Volunteers were recruited from patients attending the adult diabetic out-patient clinic at the University Hospital of Wales according to the following criteria: (1) age 18-30 (2) glycosylated haemoglobin (HbA_{1c}) of between 7-10% (3) male (4) microalbuminuria negative and (5) absence of diabetic complications. Control subjects had no known physician diagnosed disease or family history of diabetes assessed by a medical history questionnaire. All subjects were non-smokers and subjects taking antioxidants were excluded.

Table 6.0 – Age and physiological characteristics of subjects

Dependent variable	*Diabetic group (n =12)* *(Range)*	*Control group (n = 13)*
Age (yrs)	26 ± 5	22 ± 3.4
Stature (cm)	182 ± 8.8	179 ± 7.5
Body mass (kg)	84 ± 14.2	81.5 ± 9.7
Body mass index (kg/m^2)	25.7 ± 4	25.3 ± 2.2
Duration of diabetes (yrs)	6 ± 5.1 (0.5 – 18)	-
Daily insulin dose ($U \cdot day^{-1}$)	58.9 ± 31.9 (8 – 106)	-
HbA_{1c} (%)	8.2 ± 1	5.5 ± 0.2*
Resting blood glucose ($mmol \cdot L^{-1}$)	10.6 ± 3	4.6 ± 0.2*

All values are means ± SD. HbA_{1c}, glycated haemoglobin. * $P < 0.05$, between group difference.

Experimental design

The local Medical Research Ethics Committee (Bro Taf) granted ethical approval and written informed consent was obtained from each subject prior to participation. Subjects were instructed to abstain from any dietary antioxidant supplementation for six weeks prior to experimental exercise. Subjects were instructed to refrain from exercise and alcohol for 48 h before the test and to maintain their usual dietary pattern. Dietary composition and caloric intake in the 72 h before the exercise test was recorded by means of a food diary and assessed using a standard nutritional assessment package as described in the methodology section 3.11. All diabetic subjects were instructed to refrain from their morning insulin dose and record their morning blood glucose concentration. If any subject experienced low blood glucose levels (≤ 3 $mmol \cdot l^{-1}$), they were allowed to ingest one slice of toast.

Anthropometric measures

On arrival at the laboratory, subject body mass and stature was determined as outlined in the methodology section 3.4.

Exercise protocol

All exercise tests were performed between 9 a.m. and 10 a.m. on the ward of the diabetic unit under the supervision of the same investigator and a medical doctor to help control for biological and inter analytical subject variation. Before exercise commenced a cooling fan was placed in front of the subject. Each subject was required to cycle to volitional exhaustion on a friction braked cycle ergometer (methodology section 3.8). The test was designed to be progressive and incremental in order to elicit $\dot{V}O_{2\,max}$. Cycling commenced with a 2 kg mass on the basket. A cadence of 60 $\pm$ 3 rpm was maintained while workload was increased by 0.5 kg every 3 min until volitional fatigue. This particular protocol was chosen as it previously had been used to elicit an increase in oxidative stress parameters (Ashton *et al* 1998, 1999). Validation of $\dot{V}O_{2\,max}$ was obtained if the respiratory exchange ratio (RER) was $\geq$ 1.15 at the termination of test, had a plateau in the oxygen uptake/exercise intensity relationship ($\leq$ 2 ml·kg·min^{-1}) and a heart rate value to within 10 b·min^{-1} of age predicated maximum (220 bpm-age). Oxygen uptake ($\dot{V}O_2$) was monitored at rest and continuously during exercise using an on-line automated gas analysis system as described in methods section 3.6.1. Heart rate using a short-range telemetry device and rating of perceived exertion was continuously recorded as described in methods sections 3.5.1 and 3.9 respectively.

Venous blood sampling

Pre- and post-exercise supine blood samples were collected following a 12 hr overnight fast at the same time of day using the vacutainer method as outlined in methodology sections 3.3.1 and 3.3.2.

PBN adduct preparation and analysis

Fresh PBN was prepared in the morning of experimental day. The PBN adduct was analysed and the spectra measured on a Bruker EMX X-band ESR spectrometer. A detailed description of PBN preparation and adduct analysis can be obtained by referring to methodology section 3.2.7.

LIPID PEROXIDATION MEASURES

Malondialdehyde (MDA) analysis

MDA was measured by HPLC in EDTA plasma using a modified method of Young and Trimble (1991). A detailed description can be obtained by referring to methodology section 3.3.6.1.

Lipid hydroperoxide (LH) analysis

LH was measured spectrophotometrically in serum using a modified method of Wolff (1994) and Nourooz-Zaheh *et al* (1994) (***F***errous ***O***xidation of ***X***ylenol orange method; FOX 1). A detailed description can be obtained by referring to methodology section 3.3.6.2.

ANTIOXIDANT STATUS

Ascorbic acid analysis

Ascorbic acid was measured in EDTA plasma using the method of Vuilleumier and Keck (1989). A detailed description can be obtained by referring to methodology section 3.3.7.1.

α-tocopherol, retinol, lycopene, α- and β-carotene analysis

The HPLC method of Catignani and Bieri (1983) and Thurnham *et al* (1988) was used to stimultaneously determine plasma lipid soluble antioxidant status. A detailed description can be obtained by referring to methodology section 3.3.7.2.

Blood glucose analysis

Blood glucose was measured by dry chemistry slide technology. A detailed description can be obtained by referring to methodology section 3.3.11.

Glycosylated haemoglobin (HbA_{1c}) analysis

The HPLC method of Philcox *et al* (1992) was used to analyse HbA_{1c} percentage. A detailed description can be obtained by referring to methodology section 3.3.12.

Plasma volume assessment

Blood haemoglobin and packed cell volume were measure in order to calculate the change in plasma volume (Dill and Costill, 1974; refer to methodology sections 3.3.4 and 3.3.5).

Statistical analysis

Statistical analysis was performed using the SPSS social statistics package - version 9.0 (Surrey, UK). Data were analysed using parametric statistics following mathematical confirmation of a normal distribution by repeated Kolmogorov-Smirnov tests. For a detailed description of statistical procedures refer to methodology section 3.12.3.

6.2 – RESULTS

(A) Dietary status

Table 6.1 – Nutritional profile for diabetic (n = 12) and control (n =13) groups

Variable	*Diabetic*	*Control*	*Significance*
Energy (Kcal)	2398 ± 523	2961 ± 661	**NS**
Fat (grams)	96.2 ± 31.5	120 ± 36	**NS**
Carbohydrate (grams)	333.7 ± 116	393 ± 99	**NS**
Protein (grams)	62.4 ± 32.5	101 ± 19.5	***P < 0.05***
Fibre (grams)	13.3 ± 4.6	12.8 ± 5.71	**NS**
Sugar (grams)	157 ± 98	188.7 ± 79	**NS**
PUFA (grams)	18.3 ± 10.2	20.3 ± 4.8	**NS**
Saturated fats (grams)	36.5 ± 12	50.3 ± 17.3	**NS**

Values are means ± SD. Kcal, kilocalories; PUFA, polyunsaturated fatty acids; NS, not significant; $P < 0.05$, between group difference.

There was no significant difference in caloric intake and macronutrient composition between groups, and all values were within the recommended UK daily range (Bender and Bender, 1986). However, the healthy group demonstrated a greater protein intake than the diabetic group ($P < 0.05$).

(B) Respiratory and cardiovascular data

Table 6.2 – Maximal exercise data for diabetic and control groups

Variable	*Diabetic*	*Control*	*Significance*
RER (arbitrary units)	1.29 ± 0.07	1.21 ± 0.07	***P < 0.05***
$\dot{V}O_{2\,max}$ ($ml{\cdot}kg{\cdot}min^{-1}$)	38.6 ± 6.3	40.6 ± 12.9	**NS**
HR ($b{\cdot}min^{-1}$)	192 ± 10	189 ± 14	**NS**
RPE (arbitrary units)	18 ± 2	18 ± 1	**NS**
Power output (kg)	3.36 ± 0.39	4.23 + 0.43	***P < 0.05***
Exercise time (min)	11.34 ± 1.47	16.31 ± 3.32	***P < 0.05***

Values are means ± SD. RER, maximal respiratory exchange ratio; $\dot{V}O_{2\,max}$, maximal oxygen uptake; HR, heart rate; RPE, rate of perceived exertion; NS, not significant; $P < 0.05$, between group difference.

Exercise time to exhaustion and maximal power output was significantly greater in the control group by 4.51 mins and 0.87 kg respectively ($P < 0.05$). There was a difference of 0.8 arbitrary units in RER observed between the two groups ($P < 0.05$). $\dot{V}O_{2\,max}$ and HR values did not differ between groups at exhaustion as shown in table 6.2.

Figure 6.0 – Resting oxygen uptake values for the diabetic and control groups

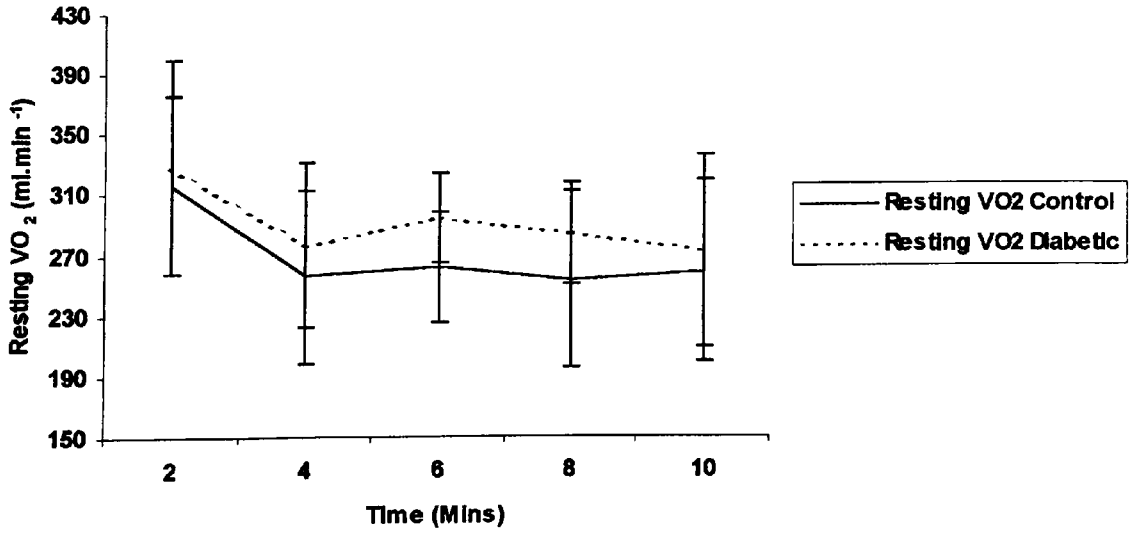


Figure 6.0 shows resting oxygen uptake values for both groups. No within or between group differences were observed.

Figure 6.1 – Exercise-induced oxygen uptake values for the diabetic and control groups

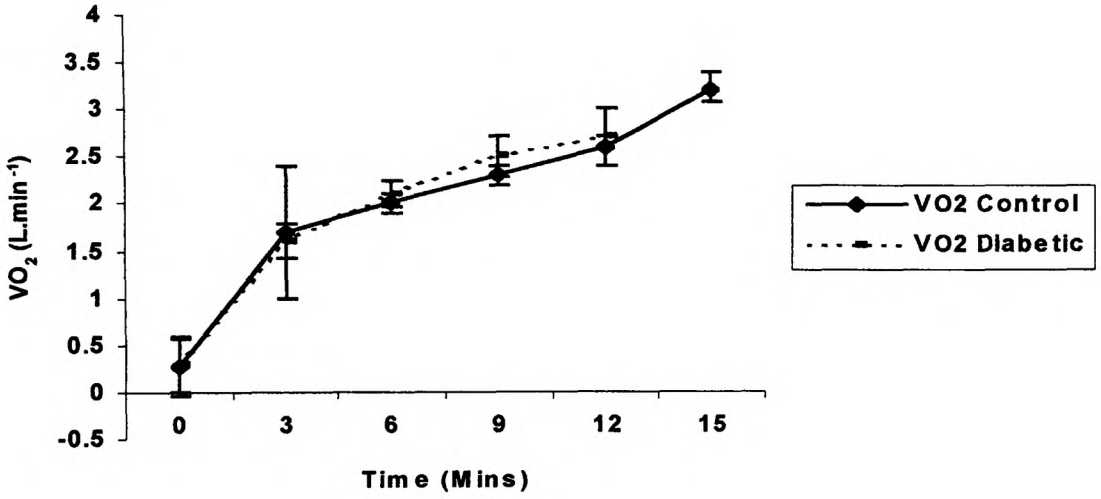


Figure 6.1 demonstrates oxygen uptake over time. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however no interaction effect was observed (*time x group*, $P > 0.05$).

(C) Oxidative stress parameters

Figure 6.2 – Rest and exercise PBN adduct concentration for the diabetic and control groups

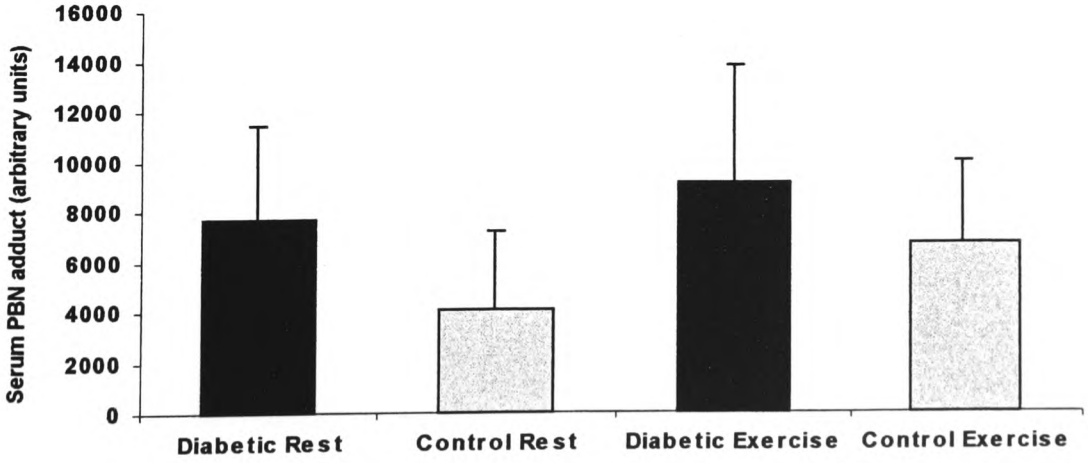
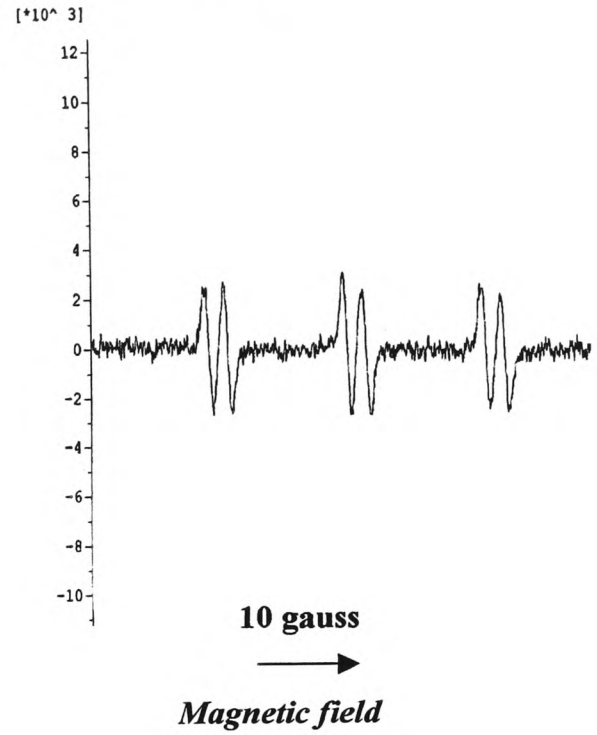


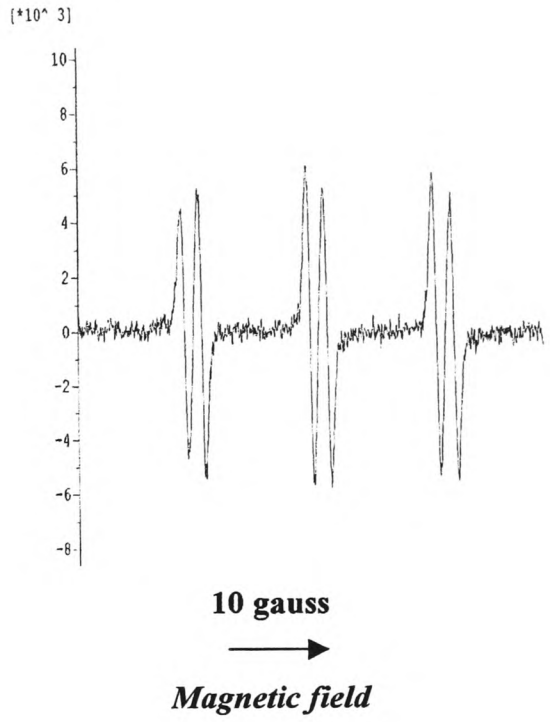
Figure 6.2 shows the effect of exhaustive exercise on free radical generation for both groups. There was a main effect for time (*rest vs. exercise*, $P < 0.05$) and group (*diabetic vs. control*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$; retrospective power calculation = 0.161). The change/difference in free radical concentration as a function of time and between groups equates to 36% and 56% respectively.

Figure 6.3 - Typical rest (A) + (B) and exercise (C) + (D) Electron Spin Resonance (ESR) spectra of α-phenyl-tert-butylnitrone (PBN) adducts in control and diabetic serum.

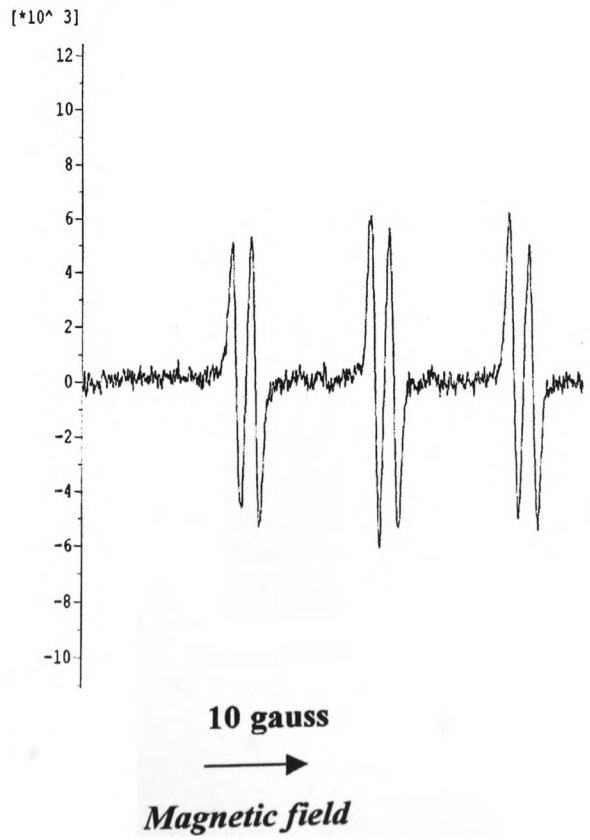
***Rest* - (A) Healthy**



(B) Diabetic



***Exercise* - (C) Healthy**



(D) Diabetic

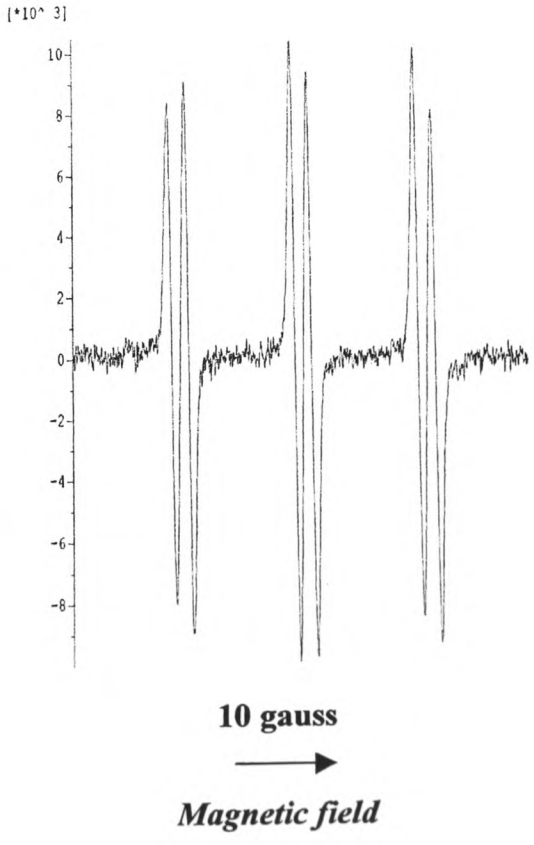


Figure 6.4 – PBN adduct concentration normalised for glycemic control (HbA_{1c}) for rest and exercise diabetic and control values

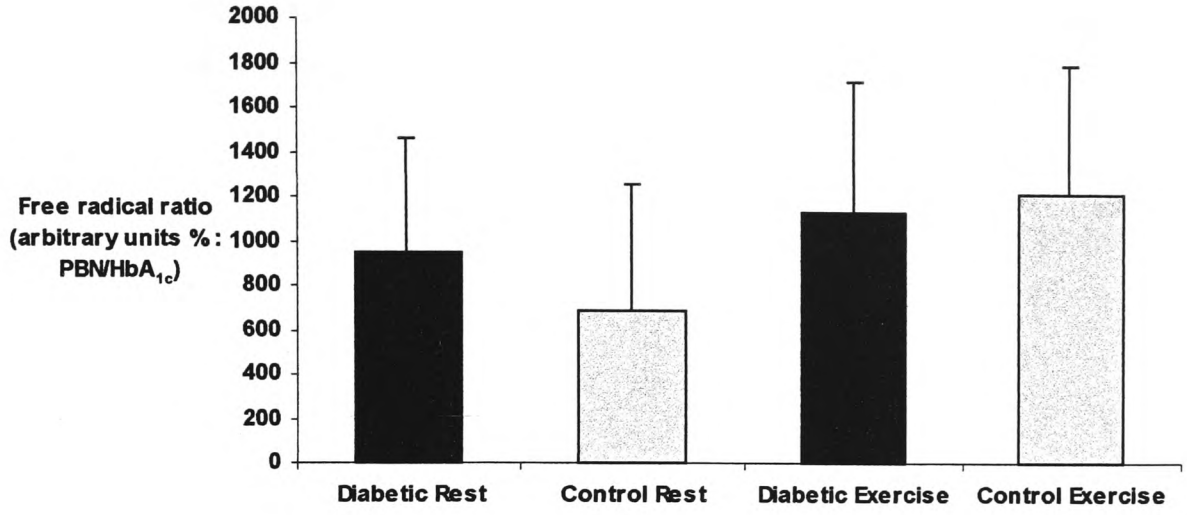


Figure 6.4 shows the PBN adduct concentration normalised for glycemic control. There was a main effect for time (*rest vs. exercise*, $P < 0.05$), however, no effect for group (*diabetic vs. control*, $P > 0.05$) or interaction effect was observed (*time x group*, $P > 0.05$).

Figure 6.5 – Rest and exercise lipid hydroperoxide (LH) concentration for the diabetic and control groups

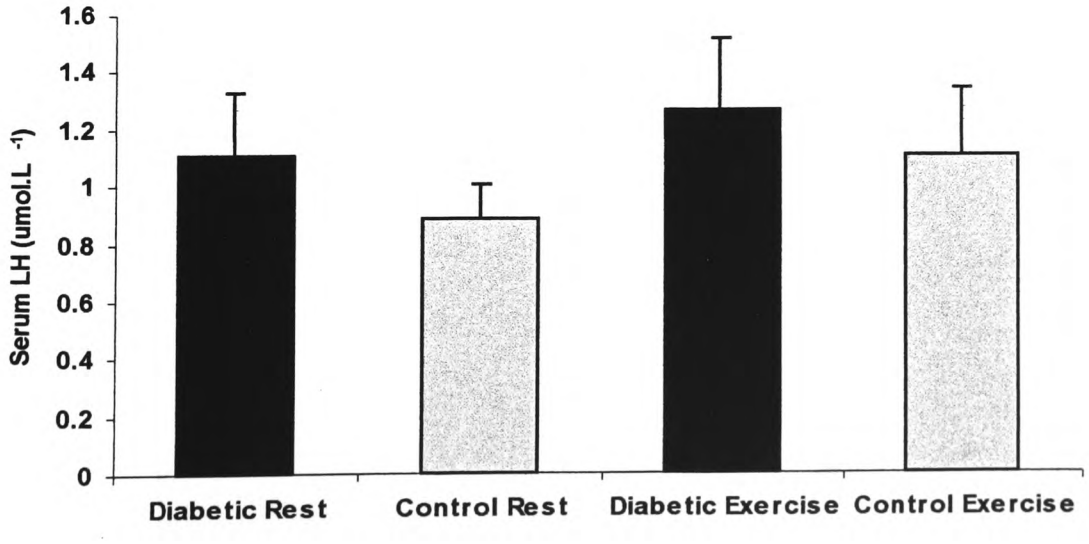


Figure 6.5 demonstrates the effect of exhaustive exercise on lipid hydroperoxide concentration for both groups. There was a main effect for time (*rest vs. exercise*, $P < 0.05$) and group (*diabetic vs. control*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$). The change/difference in lipid hydroperoxide concentration as a function of time and between groups equates to 19% and 19% respectively.

Figure 6.6 – Lipid hydroperoxide (LH) concentration normalised for glycemic control (HbA_{1c}) for rest and exercise diabetic and control values

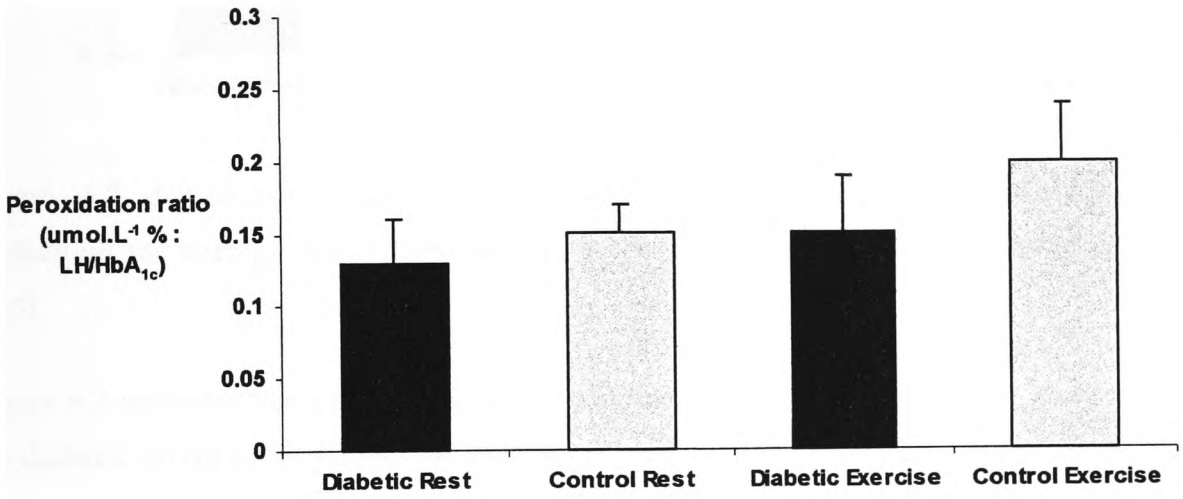


Figure 6.6 shows the lipid hydroperoxide concentration normalised for glycemic control. There was a main effect for time (*rest vs. exercise*, $P < 0.05$) and group (*diabetic vs. control*, $P < 0.05$), however, no interaction effect was observed (*time x group*, $P > 0.05$).

Figure 6.7 - Rest and exercise malondialdehyde (MDA) concentration for the diabetic and control groups

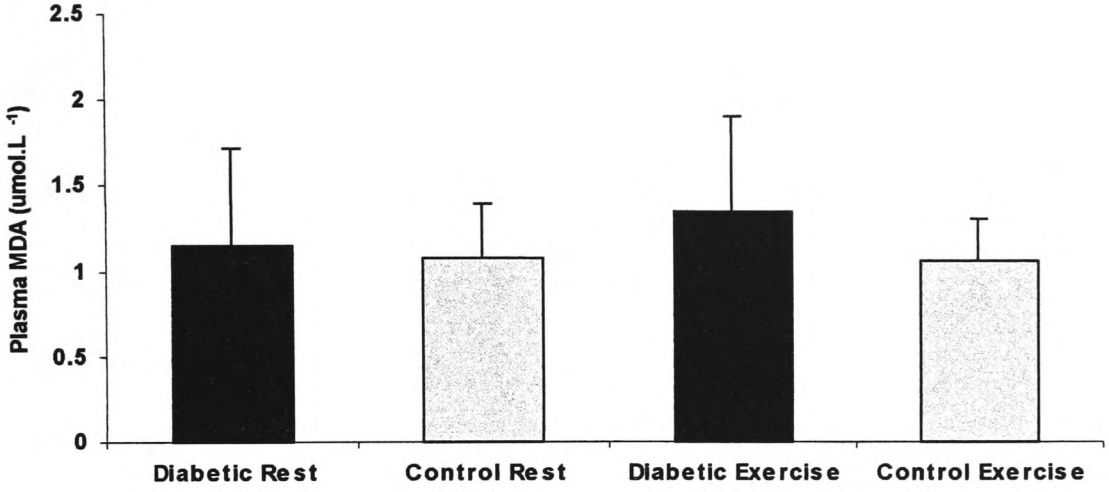


Figure 6.7 demonstrates the effect of exhaustive exercise on malondialdehyde production for both groups. There was no within or between group differences ($P >$ 0.05).

Figure 6.2 indicates that the concentration of PBN adduct was comparatively greater in the diabetic group (pooled rest and exercise data, $P < 0.05$ *vs. control group*). Typical ESR spectra at rest and exercise in control and diabetic serum are shown in figure 6.3. For all samples the hyperfine coupling constants were a_N = 13.7 gauss and $a\beta_H$ = 1.7 gauss, which are identified as being secondary oxygen-centred alkoxyl lipid radicals. The LH response was also comparatively greater in the diabetic group (pooled rest and exercise data, $P < 0.05$ *vs. control group*) as shown in figure 6.5, whilst no differences were observed in MDA as shown in figure 6.7. Physical exercise increased both the PBN adduct and LH concentration (pooled diabetic and control values, $P < 0.05$), but were not selectively different between groups. When the data was normalised for glycemic control (HbA_{1c}), physical exercise increased both the PBN adduct and LH concentration (pooled diabetic and control values, $P < 0.05$), however, LH was comparatively less in the diabetic group (pooled rest and exercise data, $P < 0.05$ *vs. control group*), whilst no difference was observed for PBN adduct concentration between groups.

(D) Antioxidant activity

Table 6.3 – Selected antioxidant indices at rest and exercise for the diabetic and control groups.

Group	*Diabetic*		*Control*	
Time	*Rest*	*Exercise*	*Rest*	*Exercise*
Variable				
α-tocopherol	20.69 ± 5.9	21.18 ± 3.8	16.93 ± 2.5	15.49 ± 3.1*†
Main effect for group				
Interaction effect for time x group				
Ascorbic acid	32.96 ± 16.1	34.77 ± 13.5	31.2 ± 3.8	31.2 ± 7.3
NS				
Retinol	0.52 ± 0.24	0.47 ± 0.24	0.76 ± 0.12	0.68 ± 0.12
Main effects for time and group				
Lycopene	0.14 ± 0.05	0.12 ± 0.09	0.15 ± 0.06	0.13 ± 0.05
NS				
α-Carotene	0.02 ± 0.01	0.01 ± 0.01	0.02 ± 0.01	0.01 ± 0.01
Main effect for time				
β-Carotene	0.05 ± 0.024	0.03 ± 0.02	0.05 ± 0.02	0.05 ± 0.02
NS				

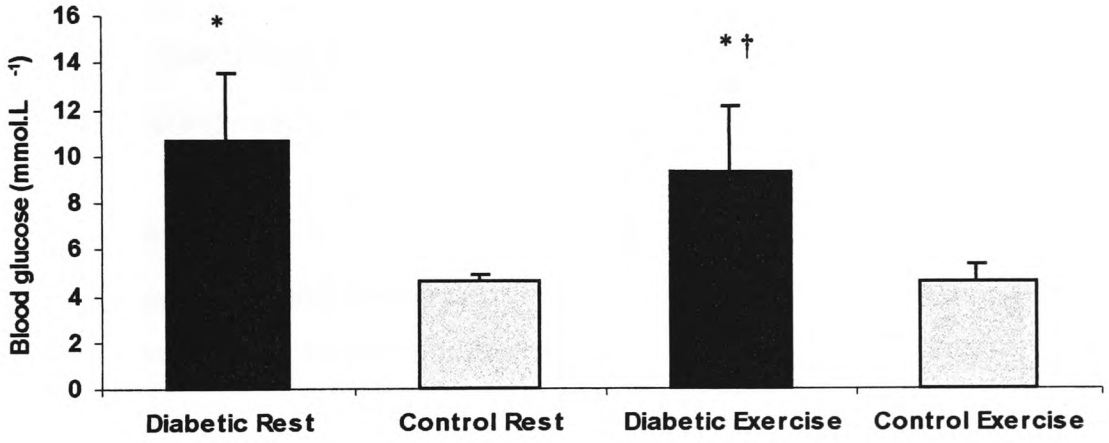
Values are means ± SD, and expressed as $\mu mol \cdot L^{-1}$; n = 13 (control group), n = 12 (diabetic group). NS, not significant; main effect for *time* indicates a difference ($P < 0.05$) between *rest vs. exercise* (pooled diabetic and control values); main effect for *group* indicates a difference ($P < 0.05$) between *diabetic vs. control* (pooled rest and exercise values); interaction effect (*time x group*) indicates a difference ($P < 0.05$) within/between groups. * between group difference as a function of time ($P < 0.05$); † within group difference ($P < 0.05$).

The venous concentration of α-tocopherol was comparatively lower (*29% difference*) in the control group (pooled rest and exercise data, $P < 0.05$ *vs. diabetic group*) due to a selective decrease during physical exercise ($P < 0.05$). In contrast, plasma retinol concentration was comparatively lower (*45.4% difference*) in the diabetic group

(pooled rest and exercise data, $P < 0.05$ *vs. control group*). Physical exercise *per se* decreased both retinol (*by 11.3%*) and α-carotene (*by 100%*) concentrations (pooled diabetic and control values, $P < 0.05$). No between or within group differences were observed for ascorbic acid, lycopene or β-carotene.

(E) – Blood glucose

Figure 6.8 – Rest and exercise blood glucose concentration for the diabetic and control groups



* indicates difference between group (*diabetic vs. control*, $P < 0.05$), † indicates difference within group (*rest vs. exercise*, $P < 0.05$) as a function of time.

Figure 6.8 demonstrates the effect of exhaustive exercise on blood glucose concentration for both groups. There was a main effect for *time* (*rest vs. exercise*, $P < 0.05$) and *group* (*diabetic vs. control*, $P < 0.05$), and a *time x group interaction* ($P < 0.05$). Venous blood glucose concentration was comparatively higher in the diabetic group (pooled rest and exercise data, $P < 0.05$ *vs. control*), due to differences in both resting ($P < 0.05$) and post-exercise values ($P < 0.05$). Physical exercise *per se* decreased blood glucose concentrations (pooled diabetic and control values, $P < 0.05$), due mainly to a selective decrease post-exercise in the diabetic group ($P < 0.05$).

Experimental inter-assay correlations

Please see methodology section 3.12.3 for explanation of correlations.

In the present investigation, a positive correlation was observed between the Δ PBN adduct and Δ LH in the diabetic group only ($r = 0.58$, $P < 0.05$). In the same group a negative relationship was found between insulin dosage and Δ LH concentration ($r = -0.65$, $P < 0.05$).

Although there were no within or between group differences for malondialdehyde concentration, there was a positive association between the Δ PBN adduct and Δ MDA in the control group only ($r = 0.58$, $P < 0.05$).

Δ $\dot{V}O_2$ correlated inversely with Δ α-carotene ($r = -0.63$, $P < 0.05$) in the control group, but not in the diabetic group. In addition, an inverse correlation was also observed between Δ LH and Δ lycopene in this group ($r = -0.55$, $P < 0.05$).

In agreeing with previous reports (Santini *et al* 1997, Gallou *et al* 1993, Griesmacher *et al* 1995) no association was found between HbA_{1c} and any of the oxidative stress markers (LH, $r = 0.9$, $P > 0.05$; PBN adduct, $r = 0.24$, $P > 0.05$; MDA, $r = 0.32$, $P > 0.05$). Nor was there any relationship between duration of diabetes and oxidative stress (LH, $r = 0.77$, $P > 0.05$; PBN adduct, $r = 0.50$, $P > 0.05$; MDA, $r = 0.29$, $P > 0.05$).

6.3 - DISCUSSION

Strong experimental evidence indicates that patients with type 1 diabetes mellitus are susceptible to increased blood levels of oxidants, confirmed mainly from indirect by-products of lipid peroxidation (Santini *et al* 1997, Griesmacher *et al*, 1995). This study demonstrates for the first time that male type 1 diabetic patients have a higher concentration of free radical species than healthy controls, as measured directly by ESR spectroscopy. The diabetic patients also had increased lipid hydroperoxide values, despite a higher venous α-tocopherol concentration. However, once the PBN adduct

and lipid hydroperoxide values were normalised for glycemic control (using HbA_{1C}), lipid hydroperoxides were comparatively less in the diabetic group with no change observed for PBN adduct concentration, suggesting that oxidative stress in the diabetic group is controlled to a large extent by glucose auto-oxidation. Results also demonstrate increased circulating levels of lipid hydroperoxide and PBN adduct formation as a function of exhaustive exercise, however no selective difference was observed between groups.

Oxidative stress and antioxidant activity in type 1 diabetes mellitus

The hyperfine coupling constants for all *ex vivo* PBN trapped radicals (a_N = 13.7 gauss and $a\beta_H$ = 1.7 gauss) suggest that the species detected in the present study are oxygen-centred lipid alkoxyl radicals, possibly derived from oxidative damage to cellular membranes. It is thought that these species are formed well down the chain of events that typifies lipid peroxidation, and are likely to be secondary or even tertiary radicals. The author can be confident that the radicals detected are not from glycosylated proteins, since Finotti *et al* (2001) show different hyperfine coupling constants (a_N = 14.0 gauss and $a\beta_H$ = 2.2 gauss) from incubated serum albumin glycated with 25 mM and 50 mM of glucose. It is worth mentioning that the free radical concentration was greater in 50 mM of glucose in comparison to 25 mM, thus implicating glucose as a potential contributor towards free radical production. These authors further suggest the radicals detected are carbon-centred species, originating from the oxidative modification of proteins. The coupling constants observed in the present study compare favourably with previous reports (a_N = 13.7 gauss and $a\beta_H$ = 1.9 gauss, Ashton *et al*, 1998), and to the work of Tortolani *et al* (1993) (a_N = 13.6 gauss and $a\beta_H$ = 1.9 gauss) examining patients undergoing elective cardioplegia.

A previous report concerned with free radical generation in type 2 diabetes has measured PBN adducts extracted with chloroform following a high fat meal (Anderson *et al*, 2001). These scientists report hyperfine coupling constants (a_N = 13.9 gauss and $a\beta_H$ = 2.2 gauss) similar to those observed in the present study, and are identified as being alkoxyl radicals derived from lipid hydroperoxide decomposition in the extracellular compartment.

It is hypothesised that the greater concentration of free radical species detected in the diabetic group may be a consequence of primary free radical attack to erythrocyte membranes or circulating lipids, initiating polyunsaturated fatty acid (PUFA) breakdown, and generating alkoxyl intermediates from lipid hydroperoxide decomposition (Clemens and Waller 1987, Duthie 1993). In support, both Tortolani *et al* (1993) and Bolli *et al* (1989) attribute the detection of alkoxyl radical formation to initial oxygen-centred radical attack on membrane phospholipids. Evidence is provided for the generation of peripheral oxidative damage by Toborek and Henning (1994) who showed that fatty acids cause an increase in oxidative stress in cultured endothelial cells.

Alkoxyl free radicals are formed as a result of lipid hydroperoxide decomposition (Anderson *et al* 2001, Tortolani *et al* 1993). The diabetic patients in this study presented a greater lipid hydroperoxide concentration in comparison to the control group. This finding partially confirms that the origin of the PBN adduct is lipid derived, and is further supported by the positive association observed between the delta PBN and lipid hydroperoxide concentration. This claim is in general agreement with previous work (Ashton *et al* 1998, Anderson *et al* 2001, Tortolani *et al* 1993). Thus, increased detection of alkoxyl free radicals and lipid hydroperoxide concentration strongly supports the presence of increased oxidative damage to lipid membranes in diabetes (Anderson *et al*, 2001).

In support, other reports show elevated lipid membrane peroxidation in diabetes. For example, Santini *et al* (1997) demonstrates increased plasma levels of lipid hydroperoxides when compared with nondiabetic subjects. This study is particularly applicable to the present investigation, as they where interested in the oxidative stress levels of young, uncomplicated type 1 diabetic subjects with average metabolic control. Moreover, Bono *et al* (1987) have detected an increase in red cell membrane-protein-sulphydryl content in the erythrocyte membrane of diabetic patients. In addition to the current work carried out in diabetes, others suggest increased oxidative stress levels as assessed by lipid membrane damage. A recent study published has shown that F_2-isoprostanes are higher in patients with Crohn's disease in comparison to healthy subjects (Wendland *et al*, 2001). Although this study did not measure free radical activity directly, this finding confirms that increased oxidative stress is not confined to the pathogenesis of diabetes mellitus.

It is further postulated that the primary initiating oxygen radical is superoxide anion ($O_2^{\cdot-}$), formed as a result of glucose auto-oxidation within the peripheral circulation (Gillery *et al* 1988, Ceriello *et al* 1991) and/or derived via 'leakage' from the ubiquinone-cytochrome b region of the mitochondrial electron transport chain during increased oxygen flux brought about by exercise (Davies *et al,* 1982). However, since there was no difference observed in $\dot{V}O_{2\,max}$ between the two groups, there is a possibility that glucose auto-oxidation rather than mitochondrial oxygen flux contributed towards the predominant source of $O_2^{\cdot-}$ in the diabetic group. Alessio *et al* (2000) has recently shown an elevation in lipid hydroperoxides as a result of isometric exercise, suggesting an alternative source of ROS during exercise other than a mass action effect of $\dot{V}O_2$. Thus, the author suggests that the increase in oxidative stress in the diabetic group is primarily caused by an increased rate of glucose modification and subsequent $O_2^{\cdot-}$ formation (Gillary *et al,* 1988), leading to an increase in the oxidative deterioration of circulating PUFA (Chance *et al,* 1979), mainly due to a higher baseline and post-exercise systemic blood glucose concentration.

Given the significance of increased HbA_{1c} levels in the diabetic group, it has been shown that poor glucose control can influence the generation of $O_2^{\cdot-}$ in diabetic serum (Ceriello *et al,* 1991). Likewise, Griesmacher *et al* (1995) found that diabetic patients with high HbA_{1c} (> 6.5%) values also had increased lipid peroxidation levels, however this positive association was not replicated in the present study. The diabetic group also had a lower plasma retinol concentration, which would encourage the generation of radical species (Keys and Zimmerman, 1999). This result is in agreement with the work of Young *et al* (1991) where an increase in lipid peroxidation was observed in the presence of decreased concentrations of retinol in streptozotocin treated rats.

Perhaps however, the most important evidence presented in this chapter to support the role of glucose auto-oxidation in the generation of increased oxidative stress in the diabetic group, comes from the reduction in lipid hydroperoxide and free radical concentration when normalised for glycemic control. This suggests a clear link between increased free radical concentration, lipid peroxidation and glucose modification in diabetic blood. The negative association between insulin dosage and delta lipid hydroperoxide concentration ($r = -0.65$, $P < 0.05$) further supports this claim.

As the pathology of diabetes is characterised by high blood glucose levels, it is plausible that the extracellular protein, superoxide dismutase may become inactivated through a process of glycosylation (Halliwell and Gutteridge, 1999). If this were to be the case, $O_2^{\cdot-}$ radicals formed as a result of glucose auto-oxidation would not be decomposed into H_2O_2 and would therefore increase in concentration to cause maximum disruption to the cell membrane. The presence of a high blood glucose and lipid hydroperoxide concentration in the diabetic group would indirectly provide credence to this claim. Furthermore, in an elegant study by Arai *et al* (1987), it has been shown that human erythrocyte CuZn superoxide dismutase activity gradually decreases when incubated with glucose.

What is more, oxidative stress has been hypothesised to impair insulin action through a change in the physical state of the membrane of target cells, thus causing an impairment to insulin-mediated glucose uptake (Paolisso and Giugliano, 1996). The increase in lipid hydroperoxide concentration in the diabetic group would suggest cellular membrane disruption, hence a greater availability of systemic blood glucose concentration for auto-oxidation purposes.

Within skeletal muscle the oxidation of fuel changes dramatically from a period of rest to exercise. At rest, skeletal muscle accounts for approximately 15%-20% of the total peripheral glucose utilisation, however during a bout of physical exercise such as cycling with increasing intensity and duration, the leg muscle demands in excess of 85% of the total body glucose utilisation (Hargreaves, 1995). Glucose may be derived from the liver and intramuscular glycogen stores and the rate of glucose oxidation can be measured using the Respiratory Exchange Ratio (RER) (Hargreaves 1995, Brooks *et al* 1996). Since the RER was higher in the diabetic group, this would suggest an increased rate of intracellular glucose oxidation and possible $O_2^{\cdot-}$ formation. Therefore, in addition to the proposed auto-oxidation of glucose within the systemic circulation, another possible location for *in vivo* glucose auto-oxidation, free radical generation and lipid peroxidation is presented.

During the course of a progressive exercise test to maximal exhaustion, plasma catecholamines rise exponentially with increasing workload (Hartley *et al* 1972, Mazzeo 1991). This hormonal change is particularly applicable to the present study, as

Loschen *et al* (1974) has demonstrated an increased rate of $O_2^{\cdot-}$ and H_2O_2 production from adrenaline auto-oxidation. Therefore, in addition to glucose, perhaps catecholamines may provide a source of initiating free radical species, and are thus indirectly involved in the observed increase in PBN adduct concentration in the present study.

Increased oxidative stress in the diabetic group may have other consequences. For example, the vascular dilating effects of nitric oxide may be compromised by the binding of $O_2^{\cdot-}$ to nitric oxide, leading to the formation of the damaging peroxynitrite molecule (Darley-Usmar *et al* 1995, Beckman and Koppenol, 1996); which has been associated with endothelial dysfunction (Tesfamariam, 1994). In support of this notion, Anderson *et al* (2001) showed an increase in alkoxyl radical generation with impaired endothelial function in type 2 diabetic patients. Moreover, the increase in the oxidative stress parameters in the type 2 patients was partially explained by the increased incidence of hyperglycaemia.

Investigators have suggested that perhaps physiologically controlled amounts of $O_2^{\cdot-}$ may determine "*normal*" vascular tone by controlling the presence of nitric oxide within the endothelium (Wardman 1993, Halliwell and Gutteridge 1999). It is hypothesised that the high blood glucose concentration in the diabetic group increased $O_2^{\cdot-}$ production which caused peripheral vasoconstriction (Tesfamariam, 1994). This in effect would decrease blood flow to active skeletal tissue (Saltin *et al*, 2000), thus reducing power output and exercise time to exhaustion as shown in the diabetic group.

Since haemoglobin is known to transport molecular O_2 to active skeletal muscle (Brooks *et al*, 1996), it is further proposed that due to the higher HbA_{1c} levels in the diabetic group, O_2 delivery was decreased, thereby decreasing power output and exercise time to exhaustion. Although no difference in maximal oxygen uptake data was evident between groups, this plausible biochemical explanation stems from research suggesting that when glucose binds to haemoglobin during glycosylation, this may inhibit oxygen attachment to the various polypeptide subunits, thereby decreasing whole body oxygen transport (*Personal communication, Dr T Ashton*). This mechanism is further supported by the presence of a higher blood glucose concentration in the diabetic group.

Exhaustive exercise has been shown to generate ROS and increase the circulating by-products of peroxidative damage in both the animal (Davies *et al* 1982, Jackson *et al* 1985) and human model (Ashton *et al,* 1998). The present study showed an exercise-induced increase in both free radical and lipid hydroperoxide concentrations, comparing favourably to the previous work of Ashton *et al* (1998), which described a threefold increase in alkoxyl free radical formation and a significant rise in lipid hydroperoxides following an acute bout of exhaustive exercise.

Since oxygen uptake increased significantly from rest to post-exercise, thus increasing oxygen flux in skeletal tissue (Keul *et al* 1972, Astrand and Rodahl 1986), it is suggested that increased electron flux within the mitochondrial respiratory chain enhanced electron leakage to possibly generate $O_2^{\cdot-}$ (Sen *et al* 2000, Meo and Venditti 2001). It is further suggested that these primary oxygen-centred free radical species, attacked membrane phospholipid structures leading to the formation of lipid hydroperoxide and other oxygen-centred species, such as alkoxyl free radicals (Branchaud, 1999). It is this radical that is implicated in the increased ESR signal intensity observed following exercise in the present study.

The mitochondria has long been considered a prolific source of ROS *in vivo*, and this is based on an accumulation of evidence showing production of both $O_2^{\cdot-}$ and H_2O_2 in isolated mitochondria (Boveris and Chance 1973, Loschen *et al* 1974, Boveris and Cadenas 1975, Turrens and Boveris 1980, Raha *et al* 2000). An important finding of the study by Boveris and Chance (1973) was not only did mitochondria produce ROS, but the rate of ROS generation was directly proportional to the mitochondrial oxygen utilisation. Therefore if one considers that approximately 15% of resting oxygen consumption forms ROS within the mitochondria (Sawyer, 1988), then the higher the oxygen utilisation induced by physical exercise, the greater the production of ROS by mitochondria.

It is currently considered that the cytosolic adenine nucleotides are important molecules in the control of cellular respiration (Brand and Murphy, 1987). As exercise intensity increases, ATP is degraded, whilst the increase in ADP can stimulate mitochondrial oxidative phosphorylation for energy production (Meo and Venditti, 2001). However, within mitochondrial oxidative phosphorylation, there may be a disruption in the

passing of electrons from ubisemiquinone to quinone, where electrons become "*unbound*" and diffuse through the outer membrane to form $O_2^{\cdot-}$ (Sjödin *et al* 1990, Jackson 1994). As suggested $O_2^{\cdot-}$ is possibility the initiating radical species in the present study, however $O_2^{\cdot-}$ is also known to form H_2O_2 via a reaction involving superoxide dismutase (McCord and Fridovich, 1969). As H_2O_2 is also known to generate hydroxyl radicals (Halliwell and Gutteridge, 1999), it is biochemically plausible that these ROS may exert damage to intracellular membranes. It is proposed that the lipid hydroperoxide and alkoxyl radicals generated post-exercise in this study, originated from this intracellular mechanism and diffused on formation into the venous circulation for subsequent detection.

Moreover, recent research conducted in our laboratory (Bailey *et al*, in press) has investigated localised free radical generation during single-leg quadricep exercise (intensity = 68 $\pm$ 6% WR_{MAX}). The results show a greater concentration of alkoxyl free radical species (a_N = 13.8 gauss; $a\beta_H$ = 1.8 gauss) within the femoral vein when compared to the femoral artery (venoarterial concentration difference: 361 $\pm$ 231 arbitrary units). Additionally, cycling at the same relative intensity resulted in greater ESR signals compared to isolated quadriceps exercise. This is the first data of its kind to propose that localised skeletal muscle tissue may generate lipid-derived free radicals during intensive cycling exercise which can be detected by ESR upon diffusion into the extracellular medium. These findings support the mitochondrial mechanism proposed for free radical generation in the present study.

Although this author has concentrated on the mitochondria as the predominant mechanism involved in the generation of the primary free radical species during exercise. There are however, other mechanisms that may be involved in the exercise-induced oxidative stress observed in the present study.

For example, as blood was taken from all subjects immediately post-exercise (*within approximately 60 seconds*), there is a possibility that xanthine oxidase may be involved in the generation of primary initiating free radical species. It is suggested that during strenuous exercise, blood flow was decreased to various bodily organs, and redirected to active skeletal muscle, and upon cessation of exercise the return of oxygenated blood to these organs may have caused an increase in $O_2^{\cdot-}$ generation (Witt *et al*, 1992). This

mechanism circulates around the fact the xanthine dehydrogenase is converted to xanthine oxidase (*possibility by a calcium dependent reaction*) during periods of metabolic stress. Once oxygen is reintroduced, xanthine oxidase uses molecular oxygen as opposed to NAD^+ as an electron acceptor. Molecular oxygen is therefore reduced and the $O_2^{\cdot-}$ molecule is formed (Sjödin *et al,* 1990).

Additionally during periods of extensive muscular contraction, ATP regeneration is limited, which may raise intracellular calcium ions and increase ROS. This process involves the release of calcium ions from the sarcoplasmic reticulum into the myofilaments when muscular contraction is initiated. The calcium ions subsequently bind to troponin C, which changes the protein conformation. This change in protein shape is transmitted to other thin filament components such as troponin T, troponin I, tropomyosin and actin, causing the latter two filaments to interact which releases energy. Calcium ions are usually cleared from the site of contraction by an ATP-driven pump, known as Ca^{2+}ATPase (Hanninen and Atalay 1998, McComas 1996, Tortora and Grabowski 1996). However, when ATP is reduced during exhaustive exercise, calcium ions accumulate which can activate phospholipase A_2 and through a series of reactions can generate ROS in the mitochondria. Whilst this mechanism also generates MDA, the positive association observed between delta MDA and the PBN adduct in the control group, may suggest an indirect link between the above mechanism and the increased detection of free radical species in the present study.

Furthermore, as exercise such as cycling uses a combination of both eccentric and concentric muscular contractions (Jackson, 1994), one could suggest that the potential for this mechanism to generate ROS is maximised during exhaustive cycling exercise.

Laaksonen *et al* (1996) and Atalay *et al* (1997), both report increased TBARS values in type 1 diabetic patients at rest and post-exhaustive exercise. However in contrast, the present study showed no difference in MDA values between groups or as function of exercise *per se*. This finding may be attributable to different methodology, especially in the use of a more sensitive and specific assay in quantifying MDA and in the different exercise protocol employed in the present research. One other possible mechanism attributed to the lack of overall change in MDA, may be as a consequence of alkoxyl and/or lipid peroxidation termination by the available circulating concentration of

antioxidants. Decreased concentrations of retinol and α-carotene as a function of exercise and a selective decrease in α-tocopherol post-exercise in the control group would suggest that as antioxidants they are terminating by electron donation, the further progression of the lipid derived alkoxyl radicals (Niki *et al,* 1995), and thus inhibiting the production of MDA. However, what is unknown from the present research is the actual site of radical scavenging within the lipid peroxidation chain. Additionally, since MDA levels were not measured continuously during the exercise protocol, one cannot exclude the possibility of MDA redistribution between plasma and exercising tissue.

It is well established that type 1 diabetic patients have a compromised antioxidant defence system, possibly due to the increased presence of oxidative stress (Santini *et al,* 1997). In contrast, the diabetic group in this study presented with a higher α-tocopherol concentration in comparison with the control group. This may be partially due to a lower exercise-induced oxidation rate of α-tocopherol in the diabetic group and a selective decrease during physical exercise in the control group. Pincemail *et al* (1988) has shown an increase in α-tocopherol mobilisation as a result of intensive exercise, suggesting that the venous rise may be due to a lipolysis dependent effect. Unfortunately, since circulating fatty acid concentration was not measured in this study, this explanation remains speculative. Another plausible explanation for the higher plasma α-tocopherol levels observed in the diabetic group, may be due to increased rates of oxidised α-tocopherol being regenerated by ascorbic acid (Niki *et al,* 1984). Although no change was evident in ascorbic acid concentration, one cannot rule out the possibility of the ascorbyl radical being reduced back to ascorbic acid by glutathione (Meister, 1992). The selective decrease in α-tocopherol post-exercise in one group and not the other, however warrants further investigation. Furthermore, the negative association between delta $\dot{V}O_2$ and α-carotene, and delta lipid hydroperoxides and lycopene further confirms the relationship between exercise-induced oxygen uptake and oxidative stress as proposed in the present study.

6.4 - CONCLUSION

This study demonstrates for the first time that male type 1 diabetic patients have comparatively higher levels of free radical species within the systemic circulation than

healthy controls. Supporting assays of lipid peroxidation demonstrate higher values of lipid hydroperoxides in the diabetic group, however no group difference was evident for malondialdehyde concentration, suggesting increased scavenging of the alkoxyl radical by higher circulating concentrations of α-tocopherol or greater decomposition of MDA.

Although diabetic patients are clearly more susceptible to increased levels of free radicals and lipid peroxidation, one must consider the significance of oxidative stress in this pathology. The difference between the diabetic and control groups was determined at 56% and 19% for the free radical and lipid hydroperoxide concentration respectively. These data are well within the critical difference values for both indices, suggesting that the oxidative stress levels in this group of patients may not be particularly harmful. However, as there was a difference of 45.4% for retinol, which is outside the critical difference of 28.7%, it is suggested that the increase in oxidative stress is depleting the antioxidant system of important compounds beyond a critical level.

Furthermore, it is still not known whether an increase in lipid peroxidation contributes further to molecular injury. For example, other clinical studies have showed an inhibition of lipid peroxidation, but increased cellular damage, suggesting that lipid peroxidation is not important in pathology (Silverman and Santucci 1988, Halliwell and Gutteridge 1999). Although this has never been quantitatively shown in diabetes, the increase in oxidative stress in this study, whether within the critical difference or not, does not mean that diabetic patients are free from oxidative injury, and perhaps examining other oxidation products (*e.g.* DNA, protein carbonyls) may be more meaningful when examining oxidative stress in diabetes. It is further suggested that the link between diabetes and oxidative stress requires rigorous scientific experimentation in order to elucidate its importance (Halliwell and Gutteridge, 1999).

Exhaustive physical exercise is shown to increase free radicals and membrane peroxidation (although not selectively between groups) and decrease antioxidant protection, which according to Jenkins (2000) may be the basis for the development of many pathologies later in life. It is proposed that although the measured antioxidants demonstrate the ability to help scavenge the secondary/tertiary radical species, the endogenous quantities are not sufficient to prevent oxidative stress occurring, particularly in the patient group.

The free radical concentration changed as a function of exercise by 36% and the lipid hydroperoxides by 19%. These values are within the critical difference levels of 121% and 27.5% respectively. As suggested in the previous chapter, although statistical significance was observed for these metabolites, the claim that exhaustive exercise increased oxidative stress must be viewed with caution.

Although the difference in oxidative stress between groups and within exercise is within the critical difference values, Halliwell and Gutteridge (1999) claim that any increase in oxidative stress beyond the physiological needs of the human body may potentially be detrimental. It is therefore tempting to speculate that antioxidant supplementation may be necessary to control free radical generation and hence increased levels of oxidative stress in diabetes. With this in mind, the following study will attempt to address this issue by administering ascorbic acid to diabetic patients prior to exhaustive physical exercise.

Chapter 7

Study 3

Ascorbic Acid Supplementation and Oxidative Stress in Type 1 Diabetes Mellitus

7.0 – INTRODUCTION

The preceding chapter of this thesis has shown that male type 1 diabetic patients are at a greater risk of oxidative stress as measured by an increase in the ESR signal intensity and lipid peroxidation and a decrease in antioxidant status. Furthermore, exhaustive physical exercise elevates peripheral oxidative stress levels, which is in line with previous work completed in the area (Ashton *et al* 1998, 1999). As antioxidants are known to stabilise molecules with pro-oxidant effects, a logical approach would be to administer an antioxidant compound in order to establish whether a decrease in oxidative stress may be achieved in this pathology and during exhaustive exercise.

While much evidence exists for the role of free radicals in many disease states, it has also led to promising suggestions for therapeutic antioxidant approaches (Halliwell, 1987). Although cells within the body are endowed with a rich enzymatic antioxidant defence system (*i.e. superoxide dismutase and H_2O_2-removing enzymes*), it is believed to be inadequate to prevent oxidative damage completely, thus exogenous antioxidants are important in maintaining health (Halliwell 1996, McCall and Frei 1999). For example, vitamin E is known to protect against neurodegeneration, cardiovascular disease and retinopathy (Benzie 1998, Halliwell 1996), while vitamin C may protect against cardiovascular disease and some forms of cancer (*e.g.* stomach cancer). Although, this latter antioxidant has been criticised for having pro-oxidant effects *in vitro*, it is as yet uncertain that these pro-oxidant effects have any biological relevance (Halliwell, 1996).

Vitamin C (ascorbic acid) is an essential antioxidant in humans, and in sub-clinical ascorbic acid deficiency oxidative stress is evident in nearly all tissues despite sufficient concentrations of other antioxidants including: vitamin E, glutathione, catalase and superoxide distmutase (Nandi *et al*, 1997). Vitamin C is a versatile antioxidant due to its ability to react with many aqueous ROS (*e.g. peroxyl and alkoxyl radicals*) (Frei, 1994). It also forms the first line of antioxidant defence in human plasma exposed to a variety of oxidant insults (Maxwell 1995, Frei 1994). However, despite being an antioxidant with important properties, the use of vitamin C as a therapeutic agent in combating pathological oxidative stress is limited. To this authors knowledge no information is available regarding the effect of vitamin C supplementation on oxidative stress in type 1 diabetes mellitus, particularly in the context of ESR analysis and

exercise. Thus the aim of the present study is to investigate the effect of exogenous vitamin C supplementation on free radical generation before and following exhaustive exercise in type 1 diabetes mellitus patients.

7.1 – METHODOLOGY

Subject characteristics

Twelve (n = 12) male type 1 diabetic patients (*diabetic group*) and fourteen (n = 14) apparently healthy male volunteers (*control group*) were recruited for the present study (*table 7.0 for subject characteristics*). Diabetic patients were microalbuminuria negative and had no known diabetic complications. Volunteers were recruited from patients attending the adult diabetic clinic at the University Hospital of Wales, according to the criteria outlined in study 2. Control subjects were recruited from the student population of the University of Glamorgan, and had no known disease or family history of diabetes assessed by a medical history questionnaire. All subjects were non-smokers and any subjects taking antioxidants were excluded.

Table 7.0 – Age and physiological characteristics of subjects

Group (n):	***Diabetic (n = 12)***		***Control (n = 14)***	
Intervention: sub-groups	*vitamin C (n = 6)*	*placebo (n = 6)*	*vitamin C (n = 7)*	*placebo (n = 7)*
Age: (yrs)	27 ± 2.5	27.5 ± 5	24 ± 1.7	24 ± 3
Stature: (cm)	178 ± 8.6	187 ± 8.7	178 ± 7	175 ± 4
Body mass: (kg)	82 ± 15.8	94 ± 19.2	77 ± 7	74 ± 11
Main effect for group				
Body mass index: (kg/m^2)	25 ± 3.8	27.2 ± 6.5	24.4 ± 2.2	24 ± 3.1
Duration of diabetes: (yrs)	14.8 ± 9	8.3 ± 7	-	-
Daily insulin dose: ($U.day^{-1}$)	88 ± 33.6	64 ± 45	-	-
HbA_{1c}: (%)	7.7 ± 1*	8.8 ± 1*	4.8 ± 0.5	4.5 ± 0.5
Main effect for group				
Interaction effect for group x treatment				

All values are as means ± SD. HbA_{1c}, glycated haemoglobin. Main effect for *group* indicates a difference ($P < 0.05$) between diabetic *vs.* control (pooled vitamin C and placebo values); interaction effect (*group x treatment*) indicates a difference ($P < 0.05$) within/between groups. * between group difference as a function of treatment ($P < 0.05$).

Experimental design

The experimental design is the same as reported in study 2.

Ascorbic acid supplementation

The present study incorporated a randomised, single blind, placebo-controlled approach, where twelve ($n = 6$ and 6) diabetic patients and fourteen ($n = 7$ and 7) control subjects received an acute oral bolus dose of either 1000mg (*2 x 500mg*) of L-ascorbic acid

(Nova Laboratories, Wigston, UK, batch SCP g1514) or placebo (Nova Laboratories, Wigston, UK, batch SCP g1515), exactly 2 hours before performing the maximal oxygen uptake test. The two-hour period was chosen to allow blood levels to increase following the previous work of Ashton *et al* (1999) and Thompson *et al* (2001). Additionally, it has been reported that cells are saturated with vitamin C at doses of 200mg with the plasma being saturated at doses of 1000mg (Ashton *et al,* 1999). The subjects were given a small volume of water in order to ensure complete ingestion. All subjects consumed the two tablets administered under supervision.

Anthropometric measures

On arrival at the laboratory, subject body mass and stature was determined as outlined in the methodology section 3.4.

Exercise protocol

All exercise tests were performed between 9 a.m. and 10 a.m. at the University of Glamorgan, under the supervision of the same investigator to help control for biological and inter analytical subject variation. Each subject was required to cycle to volitional exhaustion on a friction braked cycle ergometer (methodology section 3.8), and is the same protocol as outlined in study 2. Oxygen uptake ($\dot{V}O_2$) was monitored at rest and continuously during exercise using an on-line automated gas analysis system as described in methods section 3.6.1. Heart rate using a short-range telemetry device and rating of perceived exertion was continuously recorded as described in methods sections 3.5.1 and 3.9 respectively.

Venous blood sampling

Pre-supplementation, pre- and post-exercise blood samples were collected following a 12 hr overnight fast at the same time of day using the vacutainer method as outlined in methodology sections 3.3.1 and 3.3.2.

PBN adduct preparation and analysis

Fresh PBN was prepared in the morning of experimental day. The PBN adduct was analysed and the spectra measured on a Bruker EMX X-band ESR spectrometer. A detailed description of PBN preparation and adduct analysis can be obtained by referring to methodology section 3.2.7.

LIPID PEROXIDATION MEASURES

Malondialdehyde (MDA) analysis

MDA was measured by HPLC in EDTA plasma using a modified method of Young and Trimble (1991). A detailed description can be obtained by referring to methodology section 3.3.6.1.

Lipid hydroperoxide (LH) analysis

LH was measured spectrophotometrically in serum using a modified method of Wolff (1994) and Nourooz-Zaheh *et al* (1994) (***F***errous ***O***xidation of ***X***ylenol orange method; FOX 1). A detailed description can be obtained by referring to methodology section 3.3.6.2.

ANTIOXIDANT STATUS

Ascorbic acid analysis

Ascorbic acid was measured in EDTA plasma using the method of Vuilleumier and Keck (1989). A detailed description can be obtained by referring to methodology section 3.3.7.1.

α-tocopherol, β-carotene, α-carotene, lycopene and retinol analysis

The HPLC method of Catignani and Bieri (1983) and Thurnham *et al* (1988) was used to simultaneously determine plasma lipid soluble antioxidant status. A detailed description can be obtained by referring to methodology section 3.3.7.2.

Blood glucose analysis

Blood glucose was measured by dry chemistry slide technology. A detailed description can be obtained by referring to methodology section 3.3.11.

Glycosylated haemoglobin (HbA_{1c}) analysis

The HPLC method of Philcox *et al* (1992) was used to analyse HbA_{1c} percentage. A detailed description can be obtained by referring to methodology section 3.3.12.

Plasma volume assessment

Blood haemoglobin and packed cell volume were measure in order to calculate the change in plasma volume (Dill and Costill, 1974; refer to methodology sections 3.3.4 and 3.3.5).

Statistical analysis

Statistical analysis was performed using the SPSS social statistics package - version 10.0 (Surrey, UK). Data were analysed using parametric statistics following mathematical confirmation of a normal distribution by repeated Kolmogorov-Smirnov tests. For a detailed description of statistical procedures refer to methodology section 3.12.3.

7.2 – RESULTS

(A) Dietary status

Table 7.1 – Nutritional profile for diabetic and control groups

Group (n):	***Diabetic (n = 12)***		***Control (n = 14)***	
Intervention: sub-groups	*vitamin C (n = 6)*	*placebo (n = 6)*	*vitamin C (n = 7)*	*placebo (n = 7)*
Energy: (Kcal)	2747 ± 597	2478 ± 453	2861 ± 622	3048 + 868
Fat: (grams)	87 ± 42	61 ± 42.5	145 ± 63.4	116 ± 51.4
Carbohydrate: (grams)	372 ± 101	355 ± 127	358 ± 94	411 ± 129
Protein: (grams)	84 ± 32	79 ± 23	67 ± 32	82.8 ± 34
Fibre: (grams)	14 ± 6	11 ± 5	16 ± 8	18 ± 8.5
Sugar: (grams)	173 ± 97	178 ± 82	195 ± 98	181 ± 101
PUFA: (grams)	24 ± 11.2	21 ± 9	28 ± 10	20 ± 7
Saturated fats: (grams)	49 ± 18.5	44 ± 16	52 ± 19	47 ± 16

Values are means ± SD. Kcal, kilocalories, PUFA, polyunsaturated fatty acids.

There were no significant differences in caloric intake and micronutrient composition between groups, and values are within the recommended UK daily range (Bender and Bender, 1986).

(B) Respiratory and cardiovascular data

Table 7.2 – Maximal exercise data for diabetic and control groups

Group (n):	***Diabetic (n = 12)***		***Control (n = 14)***	
Intervention: sub-groups	*vitamin C (n = 6)*	*placebo (n = 6)*	*vitamin C (n = 7)*	*placebo (n = 7)*
RER (arbitrary units)	1.19 ± 0.02	1.23 ± 0.1	1.16 ± 0.03	1.22 ± 0.09
NS				
$\dot{V}O_{2\,max}$ ($ml \cdot kg \cdot min^{-1}$)	35.9 ± 5.8*	36 ± 3.7*	56.3 ± 10.3	52.1 ± 4.5
Main effect for group				
Interaction effect group x treatment				
HR ($b \cdot min^{-1}$)	186 ± 6	187 ± 8	192 ± 5	180 ± 15
RPE (arbitrary units)	18 ± 1	17 ± 1	18 ± 2	18 ± 1
Power output (kg)	3.7 ± 0.41	3.75 ± 0.68	4.35 ± 0.94	4.14 ± 0.74
Main effect for group				
Exercise time (min)	12.08 ± 3.26	14.18 ± 2.8	17.44 ± 5	15.54 ± 3.2
Interaction effect for group x treatment				

Values are means ± SD. RER, maximal respiratory exchange ratio; $\dot{V}O_{2\,max}$, maximal oxygen uptake; HR, heart rate; RPE, rate of perceived exertion; NS, not significant; * indicates difference between groups ($P < 0.05$).

Table 7.2 demonstrates the maximal exercise data for both groups. Maximal oxygen uptake was comparatively higher in the control group (pooled vitamin C and placebo data, $P < 0.05$ *vs. diabetic group*) due to a selective difference in both diabetic treatment groups ($\downarrow P < 0.05$ *vs. control*). The diabetic group also had a lower maximal power output (pooled vitamin C and placebo data, $P < 0.05$ *vs. control group*). Although an interaction effect was detected for exercise time ($P < 0.05$), post hoc analysis showed no significant differences.

Figure 7.0 – Exercise-induced oxygen uptake values for the diabetic and control groups

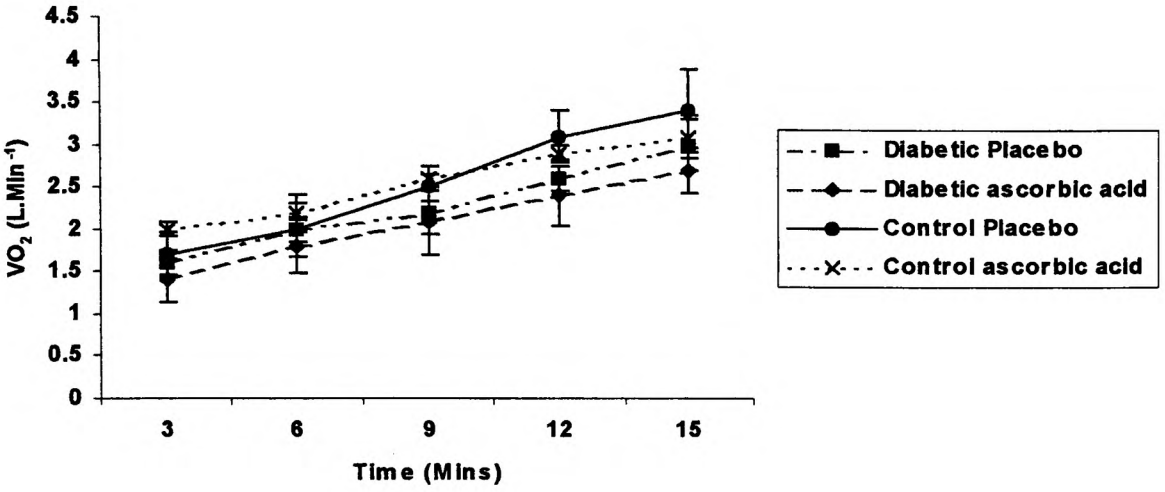


Figure 7.0 shows oxygen uptake over time. There was a main effect for time (3 *mins vs. exercise*, $P < 0.05$) suggesting that as exercise intensity increases, so does oxygen consumption. The diabetic group had a comparatively lower exercise-induced oxygen uptake (pooled vitamin C and placebo data, $P < 0.05$ *vs. control group*).

(C) Oxidative stress parameters

Figure 7.1 – Effect of ascorbic acid on rest and exercise PBN adduct concentration for the diabetic and control groups

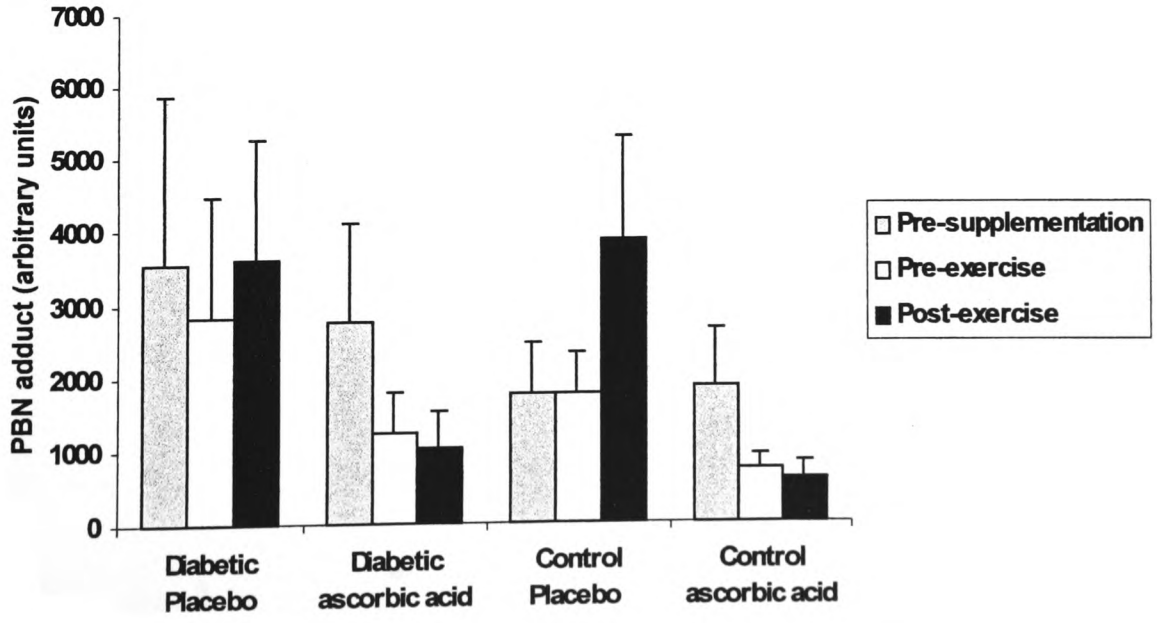
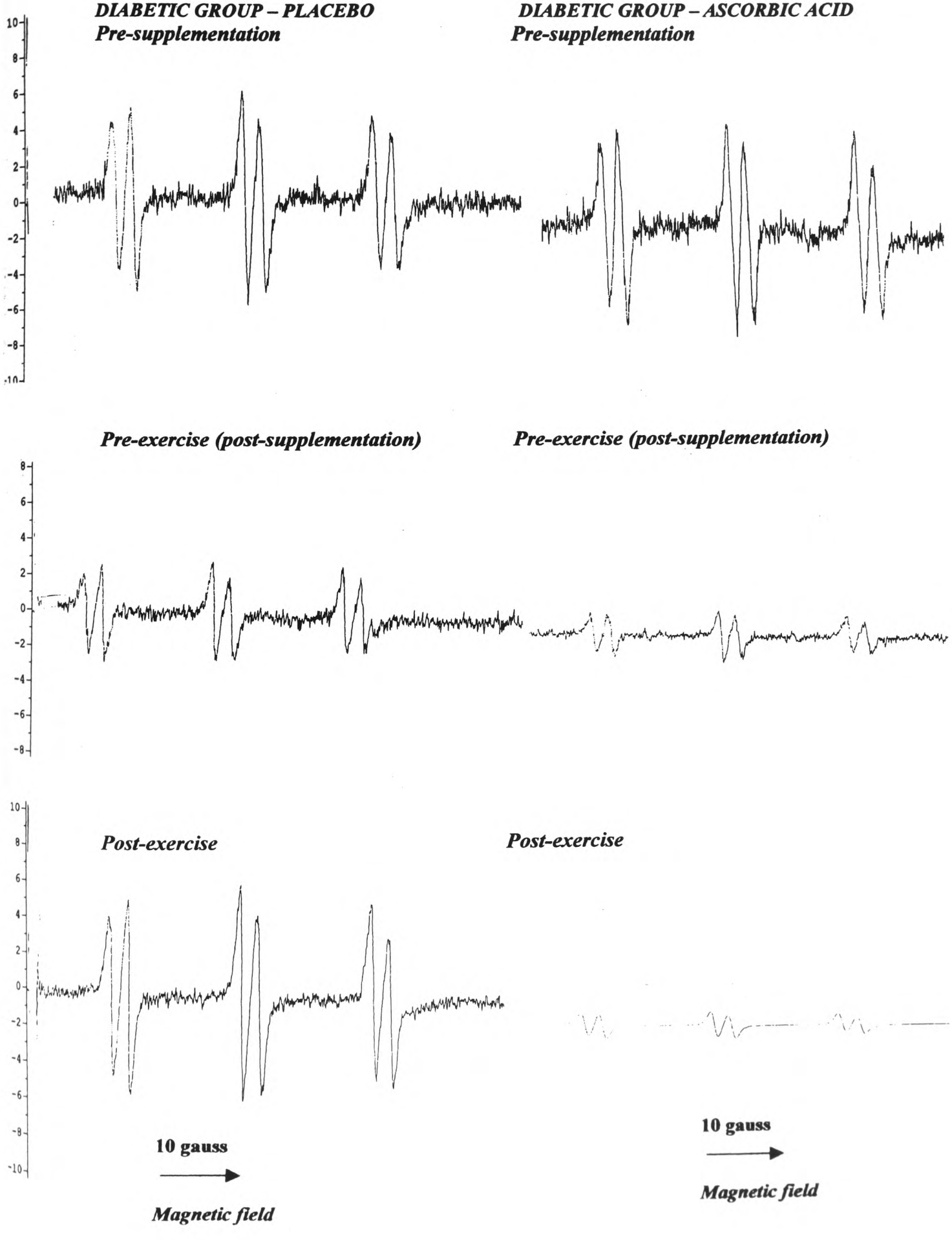


Figure 7.1 demonstrates the effect of ascorbic acid supplementation on rest and exercise free radical concentration for the diabetic and control groups. There was a main effect for time (pooled data, *pre-supplementation vs. pre-exercise vs. post exercise*, $P < 0.05$) and group (*diabetic vs. control*, $P < 0.05$). With regards to the former effect, post hoc tests between means highlighted a significant decrease ($P < 0.05$) in free radical concentration from pre-supplementation to pre-exercise, with no change as a result of physical exercise, *i.e.* vitamin C supplementation decreased resting oxidative stress. There was an interaction effect for *time x treatment* ($P < 0.05$) and *time x group* ($P < 0.05$), although no post hoc differences were detected for the latter interaction. Ascorbic acid decreased free radical concentration as evident by a main effect for treatment (*ascorbic acid vs. placebo*, $P < 0.05$). No three-way interaction effect was observed (*time x group x treatment*, $P > 0.05$; retrospective power calculation = 0.495).

Figure 7.2 – Effect of ascorbic acid on typical rest and exercise Electron Spin Resonance (ESR) spectra of α-phenyl-tert-butylnitrone (PBN) adducts in healthy and diabetic serum



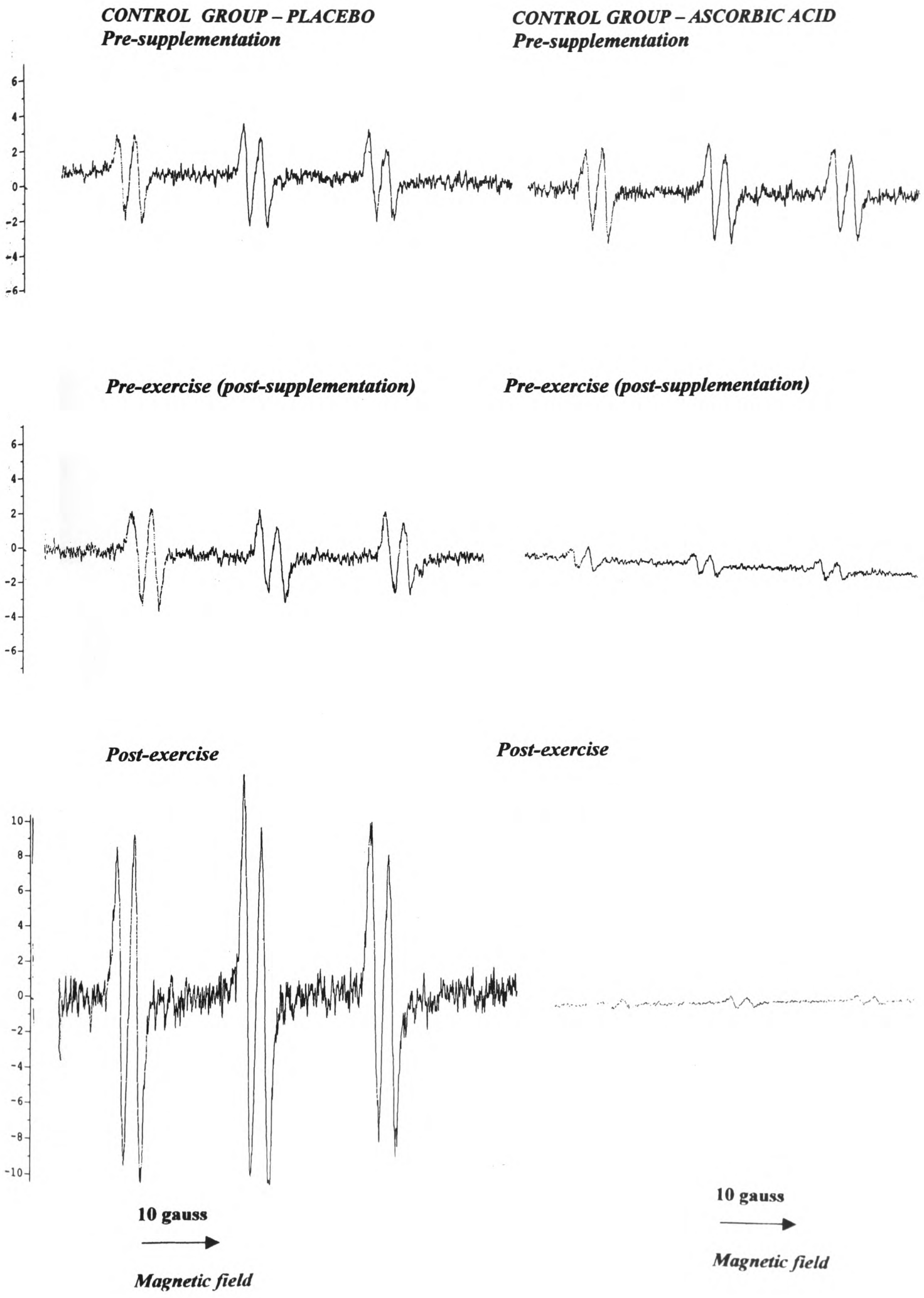

CONTROL GROUP – PLACEBO
Pre-supplementation
CONTROL GROUP – ASCORBIC ACID
Pre-supplementation
Pre-exercise (post-supplementation)
Pre-exercise (post-supplementation)
Post-exercise
Post-exercise
10 gauss
Magnetic field
10 gauss
Magnetic field

Figure 7.3 - Effect of ascorbic acid on rest and exercise lipid hydroperoxide (LH) concentration for the diabetic and control groups

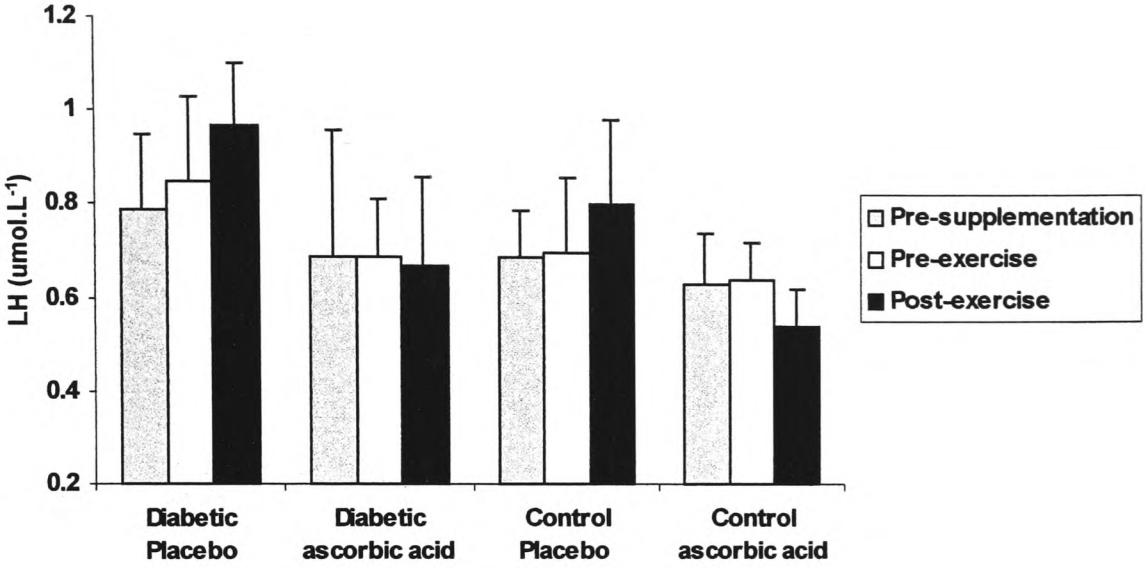


Figure 7.3 demonstrates the effect of ascorbic acid supplementation on rest and exercise lipid hydroperoxide concentration for the diabetic and control groups. There were main effects for group (↑*diabetic vs. control*, $P < 0.05$) and treatment (↓*ascorbic acid vs. placebo*, $P < 0.05$), however, no interaction effects were observed ($P > 0.05$).

Figure 7.4 - Effect of ascorbic acid on rest and exercise malondialdehyde (MDA) concentration for the diabetic and control groups

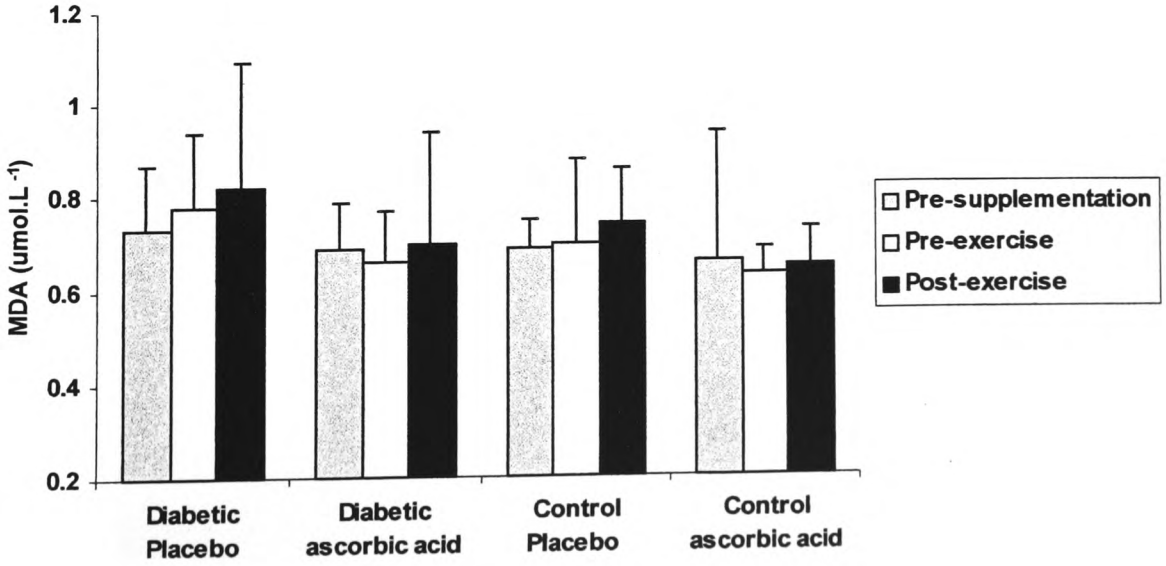


Figure 7.4 shows the effect of ascorbic acid supplementation on rest and exercise malondialdehyde concentration for the diabetic and control groups. There were no within or between group differences ($P > 0.05$).

The main oxidative stress findings include a comparatively greater free radical (*by 49%; figure 7.1*) and lipid hydroperoxide (*by 16.5%; figure 7.3*) concentration in the diabetic group (*diabetic vs. control*, $P < 0.05$). Ascorbic acid attenuated both free radical (*by 119.6%; figure 7.1*) and lipid hydroperoxide (*by 24.3%; figure 7.3*) concentration (*ascorbic acid vs. placebo*, $P < 0.05$), however these changes were not selectively different between groups (*diabetic vs. control*, $P > 0.05$). Ascorbic acid also decreased the free radicals produced during exhaustive exercise (*time x treatment*, $P < 0.05$). Typical ESR spectra showing the effects of ascorbic acid on rest and exercise PBN adduct concentration are displayed in figure 7.2. The hyperfine coupling constants for all samples were measured at $a_N = 13.7$ gauss and $a\beta_H = 1.8$ gauss, which are attributed to secondary oxygen-centred free radical species, derived possibly from lipid hydroperoxides.

(D) Antioxidant activity

Table 7.3 – Selected antioxidant indices at rest and exercise for diabetic and control groups

Group (n):	***Diabetic (n = 12)***		***Control (n = 14)***	
Intervention: sub-groups	*placebo*	*vitamin C*	*placebo*	*vitamin C*
Ascorbic acid				
Pre-supplementation	41.5 ± 13.2	43.7 ± 12.2	50.1 ± 23.5	55.8 ± 15.1
Pre-exercise	41.7 ± 16.2	102.1 ± 23.4	48.6 ± 18.8	115.6 ± 15.8
Post-exercise	45 ± 23.4	104.3 ± 25	49.2 ± 20.9	111.6 ± 22.5
Main effects for time/treatment				
Interaction effect for time x treatment				
α-tocopherol				
Pre-supplementation	21.19 ± 2	26.07 ± 8	20.47 ± 3	17.96 ± 4.1
Pre-exercise	22.5 ± 2.7	24.05 ± 7	20.31 ± 4.4	19.98 ± 3.5
Post-exercise	20.67 ± 2.8	22.76 ± 5.2	21.02 ± 3.9	19.24 ± 4.1
Main effect for group				
Retinol				
Pre-supplementation	0.76 ± 0.06	1.01 ± 0.27	1.22 ± 0.25	1.11 ± 0.43
Pre-exercise	0.83 ± 0.13	0.96 ± 0.24	1.19 ± 0.33	1.19 ± 0.37
Post-exercise	0.82 ± 0.1	0.9 ± 0.17	1.23 ± 0.30	1.23 ± 0.38
Main effect for group				
Lycopene				
Pre-supplementation	0.60 ± 0.4	0.57 ± 0.2	1.64 ± 0.8	1.3 ± 0.8
Pre-exercise	0.95 ± 0.8	0.65 ± 0.5	1.76 ± 0.75	0.77 ± 0.5
Post-exercise	0.65 ± 0.5	0.57 ± 0.3	1.25 ± 0.8	1.18 ± 1
Main effect for group				
α-carotene				
Pre-supplementation	0.12 ± 0.04	0.05 ± 0.02	0.07 ± 0.09	0.1 ± 0.06
Pre-exercise	0.12 ± 0.02	0.08 ± 0.03	0.1 ± 0.09	0.08 ± 0.03
Post-exercise	0.10 ± 0.04	0.06 ± 0.02	0.08 ± 0.09	0.11 ± 0.09
NS				
β-carotene				
Pre-supplementation	0.24 ± 0.05	0.30 ± 0.08	0.23 ± 0.1	0.33 ± 0.15
Pre-exercise	0.32 ± 0.12	0.42 ± 0.2	0.27 ± 0.1	0.23 ± 0.1
Post-exercise	0.24 ± 0.09	0.30 ± 0.1	0.22 ± 0.1	0.34 ± 0.2
Interaction effect for time x group				

All values are means ± SD, and expressed as umol·L^{-1}. NS, not significant; main effect for *group* indicates a difference between *diabetic vs. control* (pooled ascorbic acid and placebo values) ($P < 0.05$); main effect for *time* indicates a difference between *pre-supplementation vs. pre-exercise vs. post-exercise* (pooled ascorbic acid and placebo values); main effect for *treatment* indicates a difference between *ascorbic acid vs. placebo* (pooled diabetic and control) ($P < 0.05$); interaction effect (*group x treatment*) indicates a difference within/between groups ($P < 0.05$); interaction effect (*time x group*) indicates a difference in time as a function of group ($P < 0.05$).

The main antioxidant findings include a comparatively greater (*by 93%*) venous concentration of ascorbic acid in the ascorbic acid group (pooled diabetic and control values, $P < 0.05$ *vs. placebo*), due to a selective increase (*by 61.5%*) in concentration following ascorbic acid supplementation (*pre-supplementation vs. pre-exercise*, $P < 0.05$). α-tocopherol was comparatively lower (*by 15.3%*) in the control group (pooled ascorbic acid and placebo, $P < 0.05$ *vs. diabetic*). In contrast, plasma lycopene and retinol were comparatively lower (*98%* and *35.8%* respectively) in the diabetic group (pooled ascorbic acid and placebo, $P < 0.05$ *vs. control*). There was a *time x group interaction* for β-carotene ($P < 0.05$), however the post hoc test showed no significant changes ($P > 0.05$).

(E) Blood glucose

Figure 7.5 - Effect of ascorbic acid on rest and exercise blood glucose concentration for diabetic and control groups

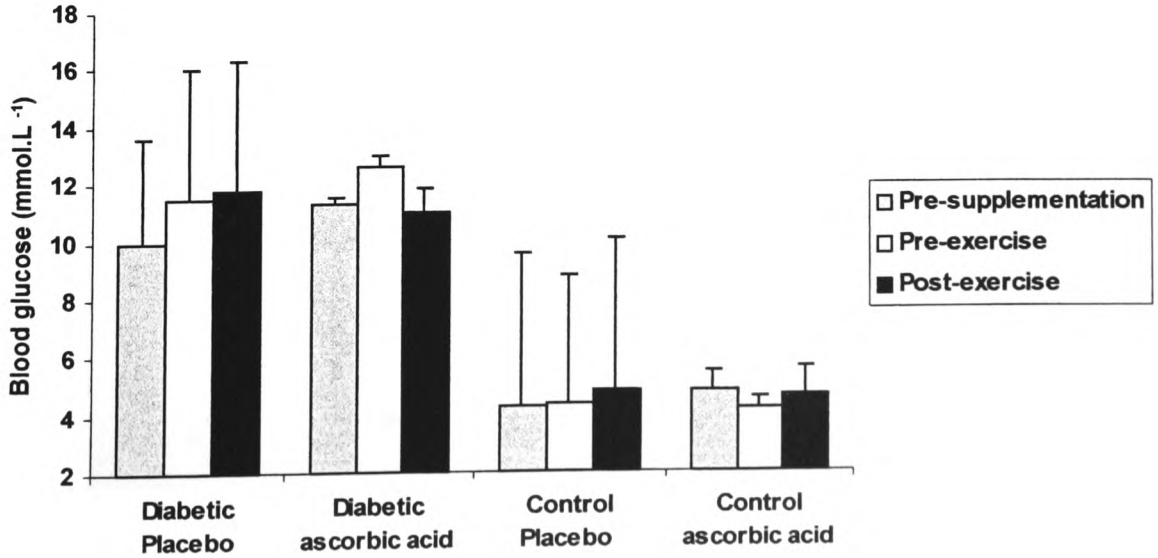


Figure 7.5 demonstrates the effect of ascorbic acid supplementation on rest and exercise blood glucose concentration for the diabetic and control groups. There was a main effect for group (↑*diabetic vs. control*, $P < 0.05$), however no interaction effects were observed ($P > 0.05$).

Experimental inter-assay correlations

Please see methodology section 3.12.3 for explanation of correlations.

ΔT_1 = pre-supplementation *minus* pre-exercise

ΔT_2 = pre-exercise *minus* post-exercise

In the present study, *positive* correlations were observed as follows:

ΔT_2 ESR and ΔT_2 lipid hydroperoxides in the diabetic placebo group ($r = 0.99$, $P < 0.05$).

ΔT_2 ESR and ΔT_2 lipid hydroperoxides in the control placebo group ($r = 0.93$, $P < 0.05$).

Negative correlations were observed as follows:

ΔT_1 ESR in the control ascorbic acid group and ΔT_1 lipid hydroperoxides in the control placebo group ($r = -0.94$, $P < 0.05$).

ΔT_2 ESR in the control ascorbic acid group and ΔT_2 ESR in the control placebo ($r = -0.84$, $P < 0.05$).

ΔT_2 ESR in the diabetic ascorbic acid group and ΔT_2 ESR in the diabetic placebo ($r = -0.91$, $P < 0.05$).

7.3 – DISCUSSION

The present study aimed to measure and quantify the effect of ascorbic acid supplementation on free radical production and lipid peroxidation in type 1 diabetes both pre- and post-exercise.

In agreement with the previous study, free radical and lipid hydroperoxide concentration was comparatively greater in the diabetic group. However, the main finding of this study is a decrease in both free radical and lipid hydroperoxide concentration following the administration of an acute oral dose of one gram of ascorbic acid; although these changes were not selectively different between the diabetic and control groups. Systemic free radical concentration was also decreased by ascorbic acid during exhaustive physical exercise. Plasma ascorbic acid concentration increased by 93% following supplementation, and in line with the previous study, α-tocopherol was also comparatively higher in diabetic plasma, although other plasma antioxidants were lower (*i.e.* retinol and lycopene).

Oxidative stress in type 1 diabetes

All hyperfine coupling constants (*in placebo and ascorbic acid supplemented groups*) measured from the ESR-PBN signal adduct were a_N = 13.7 gauss and $a\beta_H$ = 1.8 gauss, and are suggestive of secondary oxygen-centred lipid radicals. Other authors report similar hyperfine coupling constants as observed in the present study. For example, Anderson *et al* (2001) measured the PBN adducts of radicals produced in type 2 diabetic serum, and attributed the hyperfine coupling constants (a_N = 13.9 gauss and $a\beta_H$ = 2.2 gauss) to those of alkoxyl free radicals derived from the peroxidation of lipid membranes. Garlick *et al* (1987) observed values of a_N = 13.6 gauss and $a\beta_H$ = 1.5 gauss in rodent blood and suggest the species are either carbon-centred or alkoxyl radicals formed as a result of lipid degradation. This is supported by the recent work of Davison *et al* (2001) detecting free radicals (a_N = 13.6 gauss and $a\beta_H$ = 1.7 gauss) in the blood of type 1 diabetic patients. The latter authors also suggested that the radicals were derived from primary oxygen-centred damage to membrane phospholipids. Therefore, based on published work by this author and of others, it is reasonable to

suggest that the free radicals detected in the present study are lipid-derived, accumulating possibly as a result of primary oxygen-centred free radical attack to lipid membranes (Clemens and Waller, 1987).

In addition to a greater concentration of free radical species, the diabetic group also had a greater concentration of circulating lipid hydroperoxides which are known to be themselves derived from primary radicals. This in combination with the positive association between delta ESR and delta lipid hydroperoxides, helps confirm that the free radicals detected in the present investigation are lipid-derived. This is in agreement with the work of Ashton *et al* (1998) who also observed a positive correlation between lipid hydroperoxides and ESR pre-exercise ($r = 0.80$, $P < 0.05$) and post-exercise ($r = 0.71$, $P < 0.05$). These authors further suggest that the oxygen-centred alkoxyl radicals are formed via decomposition of lipid hydroperoxides, generated via primary free radical attack to cellular membranes.

Since alkoxyl radicals are known to come from phospholipid membranes (Duthie, 1993), it is postulated that the diabetic group experienced greater primary free radical attack to erythrocyte membranes or circulating lipids, yielding a greater concentration of free radical intermediates (Clemens and Waller, 1987), which are then detected by ESR spectroscopy.

Based upon evidence provided by Gillary *et al* (1988), this author suggests that the main initiating oxidising agent is $O_2^{\cdot-}$, formed as a result of glucose modification. Hunt *et al* (1990) has also shown an increase in hydroxyl radicals when hydroxylate benzoic acid was incubated with hyperglycemic levels of glucose *in vitro*. In providing support for possible increased rates of glucose auto-oxidation and subsequent free radical generation, the diabetic group in this study showed higher blood glucose levels, thus providing more substrate for auto-oxidation purposes.

In addition, it has been suggested that poor metabolic control may influence the generation of $O_2^{\cdot-}$ radicals in diabetic serum (Ceriello *et al*, 1991). This is partially supported by data presented in table 7.0 demonstrating a higher HbA_{1C} level in the diabetic group. One interpretation of this data is that abnormally high blood glucose

levels may contribute to the production of primary free radical species, which have the ability to attack lipids and generate lipid peroxides and also alkoxyl radicals.

Furthermore, Nourooz-Zadeh and co-workers (1997) have shown an increased production of lipid hydroperoxides in type 2 diabetics in the presence of a high HbA_{1C} (11 $\pm$ 2.4%). However no correlation was observed between the two variables (Nourooz-Zadeh *et al*, 1997), which supports the findings of the present study.

The diabetic group presented a greater level of oxidative stress despite having a lower $\dot{V}O_{2\,max}$. In line with chapter 6, this would suggest an alternative mechanism (for example glucose auto-oxidation) as opposed to the conventional mitochondrial source as the main contributor of initiating free radical species (Alessio *et al*, 2000). Power output was also significantly lower in the diabetic group, which coupled with a low $\dot{V}O_{2\,max}$ may reflect a relative lack of aerobic fitness in this group. Pertinent to the present findings, Laaksonen *et al* (1996) observed a strong inverse correlation between resting plasma TBARS and $\dot{V}O_{2\,max}$ in nine type 1 diabetic patients, suggesting that physical fitness/training may have a protective role in minimising oxidative stress levels in diabetes.

Although oxygen consumption increased over time, in contrast to the results presented in chapter 6, there was no increase in either free radical or lipid hydroperoxide concentration as a function of exhaustive exercise. This may be due to a relative lack of subject numbers.

Ascorbic acid supplementation and oxidative stress

Ascorbic acid supplementation lowered both free radical and lipid hydroperoxide concentration, suggesting that this antioxidant is effective in attenuating lipid peroxidation. There was however, a slight detection of free radical species and lipid hydroperoxides following 1 gram of ascorbic acid ingestion, demonstrating that ascorbic acid does not completely abolish free radical production. These findings are in general agreement with the previous work of Ashton *et al* (1999) showing an

attenuation of the ESR signal intensity after 1 gram of ascorbic acid supplementation in 10 healthy males volunteers.

As expected, plasma ascorbic acid concentration rose by 61.5% following ascorbic acid ingestion, which in is line with the recent work of Thompson *et al* (2001). However, what is unknown from the present research is the specific site of free radical scavenging. It is plausible, since plasma is saturated at doses of 1000mg, to suggest that the ascorbic acid scavenged the free radicals found in blood, thus inhibiting the propagation of lipid peroxidation (Bendich *et al*, 1986). In support of this, Frei *et al* (1989) has shown that ascorbic acid protects plasma lipids against peroxidative damage induced by aqueous lipid-derived peroxyl radicals and that ascorbic acid is the only plasma antioxidant to do so. Furthermore, Frei *et al* (1988) suggests that lipid hydroperoxides may be effectively decreased in the plasma by ascorbic acid treatment. The decreased lipid hydroperoxides in the ascorbic acid supplemented group in the present study would provide support to these claims.

It is also feasible that ascorbic acid acted intracellularly as well as in the blood, since cells are saturated at doses of 200 mg (Levine *et al,* 1996). It has been suggested that ascorbic acid is most effective at the aqueous-lipid interface of the cell membrane, scavenging intracellular aqueous radicals (Ashton *et al,* 1999). However, Thompson *et al* (2001) has recently shown that administering 1000 mg of ascorbic acid two hrs before exercise does not maximise lymphocyte cell ascorbic acid concentration. This as suggested by the authors, may reflect the conversion of ascorbic acid to dehydroascorbic acid before being transported across the cell membrane. This mechanism involves the transportation of dehydroascorbic acid into cells via the GLUT 4 protein carrier, where it is then converted back to ascorbic acid (Tsukaguchi *et al*, 1999). Ascorbic acid can also directly enter cells and is dependent on a Na^+ transporter to do so (Tsukaguchi *et al*, 1999).

In addition, lymphocyte cell ascorbic acid concentration was shown to peak during exercise of 90 mins duration (Thompson *et al*, 2001), which may suggest that the site of radical scavenging in the present study is not predominately intracellular, as a decrease in free radical concentration was observed following short-term exercise in the ascorbic acid supplemented group.

This decrease over time (*i.e.* during exercise) in free radical concentration as a function of ascorbic acid supplementation, is in agreement with the work of Ashton *et al* (1999), showing a decrease in alkoxyl radicals following exhaustive exercise.

Other potential mechanistic actions of ascorbic acid may involve the regeneration of α-tocopherol (Niki *et al*, 1984). As ascorbic acid donates an electron to regenerate the α-tocopherol radical back to α-tocopherol, ascorbyl radical intermediates may be formed. Due to the location of the unpaired electron on the ascorbyl radical, this radical is relatively unreactive, however it may interact with other free radical species to either prevent or terminate the process of lipid peroxidation (Ashton *et al*, 1999). The decrease in both aqueous phase alkoxyl radicals and lipid hydroperoxides demonstrates the effectiveness of ascorbic acid in the present study. The inverse relationships observed between the free radical concentrations in the placebo versus ascorbic acid groups, provides further support for the effectiveness of ascorbic acid within the systemic circulation.

A decreased detection of secondary free radical species and lipid hydroperoxides, may also be due to an increase in the scavenging of the primary oxygen-centred radical species by ascorbic acid. For example, there are a variety of ROS (*e.g.* $O_2^{\cdot-}$, $^1O_2$ and $OH^{\cdot}$) which may be involved in reactions with ascorbic acid (Bendich *et al* 1986, Bodannes and Chan 1979). In addition, one of the most common aqueous free radical species, the hydroperoxyl radical, is known to be scavenged by ascorbic acid (Bendich *et al,* 1986). This increased scavenging effect would decrease cell membrane damage, as observed by a decrease in lipid hydroperoxides. However, as the free radicals were reduced by 119.6% in comparison to the 24.3% in lipid hydroperoxides, it is plausible to suggest that ascorbic acid was predominately scavenging the alkoxyl radicals as opposed to the primary species.

In addition to the present finding, other studies have shown a reduction/inhibition in lipid peroxidation with ascorbic acid supplementation. Evidence suggests that ascorbic acid supplementation can protect against lipid peroxidation in atherogenic lipoproteins in human plasma (Hyyssonen *et al,* 1994). At concentrations ranging from 0.5 to 5.7 $mmol.L^{-1}$, Frei *et al* (1989) have demonstrated that ascorbic acid can protect

polyunsaturated fatty acids from oxidation. Further, dehydroascorbic acid, the oxidation product of ascorbic acid, can prevent the initiation of lipid peroxidation in LDL cholesterol (Retsky *et al,* 1993). One gram of ascorbic acid has also been shown to decrease plasma lipid hydroperoxides and malondialdehyde concentration following exhaustive exercise in humans (Ashton *et al,* 1999). Dietary vitamin C has also been shown to decrease endogenous protein damage and MDA, and maintain fatty acid unsaturation in guinea pig liver (Barja *et al,* 1994).

Antioxidant status and oxidative stress

In line with the results of the previous chapter, plasma α-tocopherol concentration was higher in the diabetic group. This would suggest that α-tocopherol is not the most effective antioxidant in preventing oxidative stress in this pathology. Although not selectively different from the control group, ascorbic acid reduced both the free radical and lipid hydroperoxide concentration in the diabetic group, confirming that ascorbic acid is the preferred antioxidant of choice and is an outstanding antioxidant in human blood plasma (Frei *et al,* 1989).

The preferential oxidation of ascorbic acid over α-tocopherol in terminating the process of lipid peroxidation may be due to the '*sluggish*' rate constant reaction of α-tocopherol with the lipid ($L^{\cdot}$) radical. Davies and Timmins (1996) show rate constant reactions of $<10^5$ $dm^3mol^{-1}s^{-1}$ and 1.3×10^7 $dm^3mol^{-1}s^{-1}$ for the interaction of α-tocopherol and ascorbic acid respectively with the $L^{\cdot}$ radical. In addition, Niki *et al* (1995) suggests that α-tocopherol cannot stabilise the alkoxyl radical before attacking PUFA, as the reaction rate constant for the former interaction is relatively slow. This author therefore postulates, that although having high concentrations of systemic α-tocopherol, the propagation of lipid peroxidation might not be prevented as the high oxidative stress indices in the diabetic group would suggest.

The relative importance of ascorbic acid in decreasing *in vivo* oxidative stress may further be highlighted by studies showing a decrease in α-tocopherol function in the presence/absence of ascorbic acid. Niki *et al* (1985) demonstrated preferential oxidation of ascorbic acid before α-tocopherol, and changes in α-tocopherol were only

observed after the depletion of ascorbic acid. It has also been shown that the antioxidant properties of α-tocopherol are maintained only in the presence of ascorbic acid (Scarpa *et al*, 1984). What is more, the α-tocopherol radical reacts rapidly with ascorbic acid encouraging the recycling of α-tocopherol (Davies and Timmins, 1996).

Another possible reason of why the diabetic group was more prone to oxidative stress may in part be due to decreased circulating concentrations of retinol and lycopene. Young *et al* (1991) has shown an increase in lipid peroxidation with a decrease in retinol concentration in streptozotocin diabetic rats. In addition to the pathology of diabetes, data from Crohn's disease patients shows a lower systemic concentration of lycopene in the presence of increased lipid peroxidation (Wendland *et al*, 2001). What cannot be determined from the present research however, is the possibility that both retinol and lycopene may also be decreased as a result of the increase in free radical concentration in the diabetic group. Although it may be determined that these antioxidants *alone* cannot protect diabetic blood from increased susceptibility to oxidative stress.

7.4 – CONCLUSION

In agreeing with the previous study, it has been established that male type 1 diabetic patients have comparatively higher (*although not critical at 49%*) levels of oxidative stress.

The present investigation has also shown that an acute oral bolus of ascorbic acid ingestion is associated with a decrease in oxidative stress, although possibly due to low subject numbers, this decrease was not selectively different between diabetic and control groups.

This author suggested in the previous chapter, that it may be necessary to supplement with antioxidants in order to control oxidative stress levels in type 1 diabetes. The present study has clearly shown a decrease in oxidative stress with ascorbic acid supplementation. However it is possible that completely abolishing free radicals may also be detrimental to health since many physiological processes in the human body rely

on free radical generation for normal function. For example, Fehrenbach and Northoff (2001) suggest that free radicals have important functions in the signal network of cells, including induction of growth and apoptosis, and as killing tools of immunocomponent cells.

This study has shown an acute attenuation by ascorbic acid of 119.6% and 24.3% for both free radical and lipid hydroperoxide concentrations respectively; which are reductions nearing critical difference levels. Although it is suggested that ascorbic acid is an effective and powerful antioxidant in human blood, further studies are warranted in order to ascertain a long-term daily ingestion dose favourable to human health, *i.e.* there may well exist a threshold or dose-response relationship between oxidative stress and normal redox function.

Chapter 8

In Vitro Studies

8.0 – INTRODUCTION

There is continuing interest in elucidating the mechanisms responsible for cell membrane damage. Determination and quantification of free radical structures becomes a starting point for clarification of the mechanisms by which cellular membranes are damaged by free radical species. The origin of free radical species, particularly within the lipid peroxidation chain, has intrigued investigators for many years (Pryor *et al* 1981, Iwahashi *et al* 1991, Stolze *et al* 2000). It is now known for example, that when a hydrogen atom is abstracted from a cell membrane polyunsaturated fatty acid (PUFA) side chain, a carbon-centred lipid radical is formed. This radical may undergo molecular rearrangement to form a conjugated diene, and in the presence of molecular oxygen this reaction may proceed to form the aqueous oxygen-centred peroxyl radical (Duthie 1993, Tappel 1973). As ascorbic acid is a known scavenger of the peroxyl radical (Frei *et al* 1989, Bendich *et al* 1986), the lipid peroxidation chain may terminate at this point. However, in the absence of sufficient ascorbic acid, lipid hydroperoxides may accumulate, and via an iron dependent reaction the oxygen-centred alkoxyl radical may be formed, which can subsequently attack aldehydes to generate malondialdehyde (Halliwell and Gutteridge, 1999).

Any reactive molecule capable of abstracting a hydrogen atom may initiate membrane lipid peroxidation, while the PUFA due to their double bond structures, are particularly susceptible to oxidative damage. Those with a greater number of double bond structures such as linoleic acid, α-linolenic acid and arachidonic acid are therefore especially vulnerable (Halliwell and Gutteridge 1999), and this has been confirmed *in vitro* by a number of investigators (Thomas *et al* 1982, Iwahashi *et al* 1996, De Groot *et al* 1973, Mason *et al* 1980).

Throughout this thesis, the author has suggested that the free radical species detected during physical exercise or pathology originate from the phospholipid membrane. However, no conclusive evidence has been provided to confirm this supposition. With this in mind, a series of *in vitro* studies were designed to [1] attempt to confirm that the free radical species detected *ex vivo* (*from an in vivo biological system*) in the human studies, originate from PUFA/membrane phosopholipids, [2] to investigate the free radical species generated from PUFA oxidation, and [3] to establish whether ascorbic

acid attenuates the detection of oxygen-centred free radical species as claimed in study 4.

8.1 – METHODOLOGY

The polyunsaturated fatty acids and total membrane phospholipid outlined in table 8.0, were chosen due to the their double bond number and thus, their susceptibility to oxidation.

Table 8.0 - number of double bonds per fatty acid molecule

Shorthand name[a]	***Common fatty acid name***
C18:2	Linoleic
C18:3	α-linolenic
C20:4	Arachidonic
Total phospholipid	L-α-phosphatidycholine

[a]Number of carbons in chain : number of double bonds (Modified from Halliwell and Gutteridge, 1999).

Experimental phase

Studies 1-4 were designed as follows:

Study 1: - Linoleic acid auto-oxidation and free radical production

Linoleic acid oxidation with/without the spin trap α-phenyl-*tert*-butylnitrone (PBN) and with/without L-ascorbic acid.

Study 2: - α-linolenic acid auto-oxidation and free radical production

α-linolenic oxidation acid with/without PBN and with/without L-ascorbic acid.

Study 3: - Arachidonic acid auto-oxidation and free radical production

Arachidonic acid oxidation with/without PBN and with/without L-ascorbic acid.

Study 4: - L-α-phosphatidycholine acid auto-oxidation and free radical production

L-α-phosphatidycholine oxidation with/without PBN and with/without L-ascorbic acid.

All of the above substrates followed the same oxidising protocol as outlined below:

1. All substrates (arachidonic acid, α-linolenic acid, linoleic acid, L-α-phosphatidycholine) and chemicals were purchased from the Sigma-Aldrich chemical company (Poole, Dorset, UK).
2. 50 µl of substrate was dissolved in 3 ml of toluene.
3. 1.5 ml of PBN (dissolved in toluene, 100mg/1.5ml) was added to this lipid-aqueous mixture. Samples without the addition of PBN were also analysed (n = 4).
4. This mixture was subsequently air auto-oxidised in a warm water bath (Clifton water baths, Nickel Electro, UK) at 37 ^{0}C for 2 hrs, after the method of Ashton (1998).
5. On completion of this incubation period, 200 µl of PBN adduct was removed, transported into a new quartz ESR glass tube and degassed before ESR analysis was performed (*methodology section 3.2.7*). The same Bruker EMX X-band ESR spectrometer that was used in the human studies was adopted for all experiments described here.

A sample from the auto-oxidation of α-linolenic acid was drawn from the mixture at 10 min, 60 min and 90 mins, and measured in an attempt to determine the rate of lipid peroxidation.

For the ascorbic acid component, the following protocol was adopted:

1. Samples were prepared as above.
2. Pure (99%) white crystalline ascorbic acid (Sigma-Aldrich, Poole, Dorset, UK) was dissolved in 3 ml de-ionised water to a concentration of 100 µmol.L^{-1}. This equates to the approximate plasma levels found in the supplemented human studies (Ashton, 1998).

3. After 2 hrs of substrate auto-oxidation, 1.5 ml of the ascorbic acid solution was added.
4. This antioxidant and pro-oxidant mixture was further oxidised for 2 hrs.
6. On completion of this incubation period, 200 µl of PBN adduct was removed and transported into a new quartz ESR glass tube and degassed before ESR analysis was performed. Identical room temperature ESR operating conditions were used for all experiments, and were as follows:

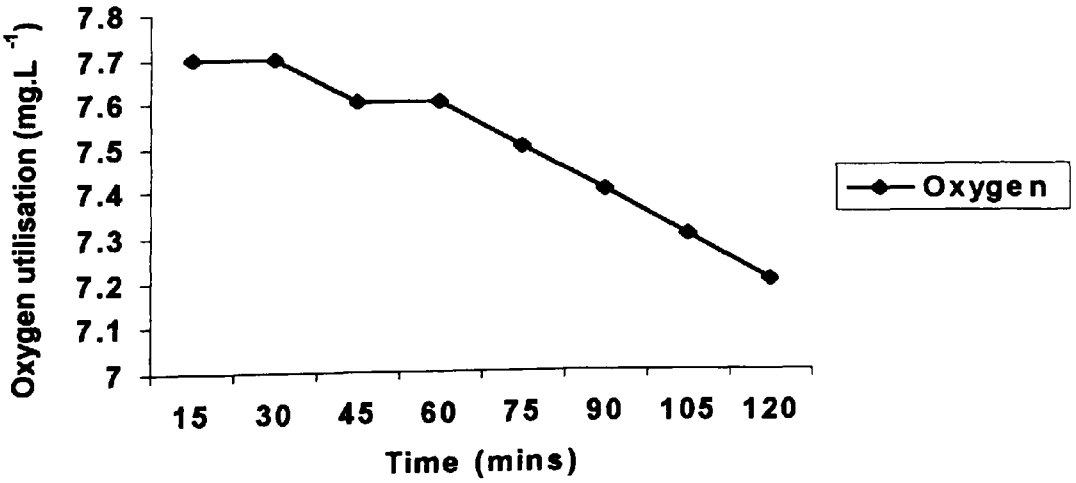
Modulation frequency, 100 kHz; microwave frequency, 9.688 GHz; incident microwave power, 20 mW; scan width, 50 gauss (*200 gauss range was initially used for location of signal and then narrowed*); modulation amplitude, 0.50 gauss; magnetic field centre, 3455 gauss; receiver gain 1.00 x 10^5; time constant, 83.9 ms; sweep time, 83.8 seconds for 10 scans.

All experiments were performed in a darkened room with minimal exposure to daylight. Oxygen uptake (O_2) was measured every 15-min period using a Clarke type oxygen electrode (Jenway, DO_2 meter, U.S.A) in linolenic acid only. Problems with the oxygen electrode membrane occurred when in contact with an organic solvent, thus further inhibiting its use.

8.2 – RESULTS

(A) Oxygen uptake data

Figure 8.0 – Oxygen uptake during α-linolenic acid air oxidation



Oxygen uptake increased over time as shown in figure 8.0. This finding may be related directly to the lipid peroxidation cascade, where oxygen is consumed in the process of peroxyl and alkoxyl radical formation.

(B) Substrate oxidation and PBN adduct data

ESR spectra, with the characteristic triplet of doublet from nitroxide PBN spin trapping, were detected in all analysis, except when each substrate was oxidised in the absence of PBN (*ESR spectra not shown*). Furthermore, and perhaps more importantly, ESR signals were not detectable following the addition of ascorbic acid to the lipid-aqueous mixture (*ESR spectra not shown*). The presence of an increased rate of α-linolenic acid oxidation and subsequent PBN adduct formation over time may be observed in figure 8.5.

Figure 8.1 – ESR spectra of PBN adduct from linoleic acid oxidation

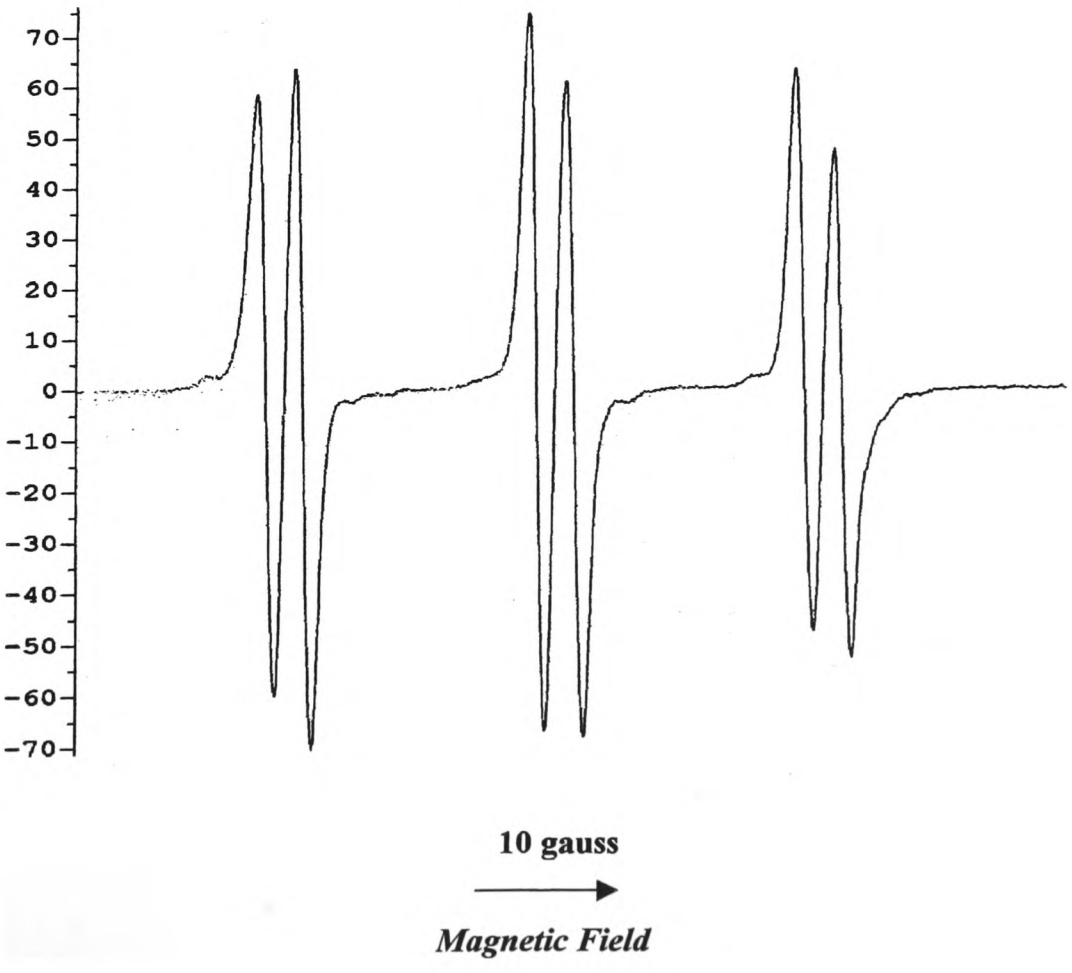


Figure 8.1 shows ESR spectra of linoleic acid oxidation. Hyperfine coupling constants for the PBN adducts were measured at a_N, 13.8 gauss; $a\beta_H$, 1.8 gauss. Mean peak amplitude height, indicating PBN adduct/free radical concentration = 121201 arbitrary units, (n = 6 peaks).

Figure 8.2 – ESR spectra of PBN adduct from α-linolenic acid oxidation

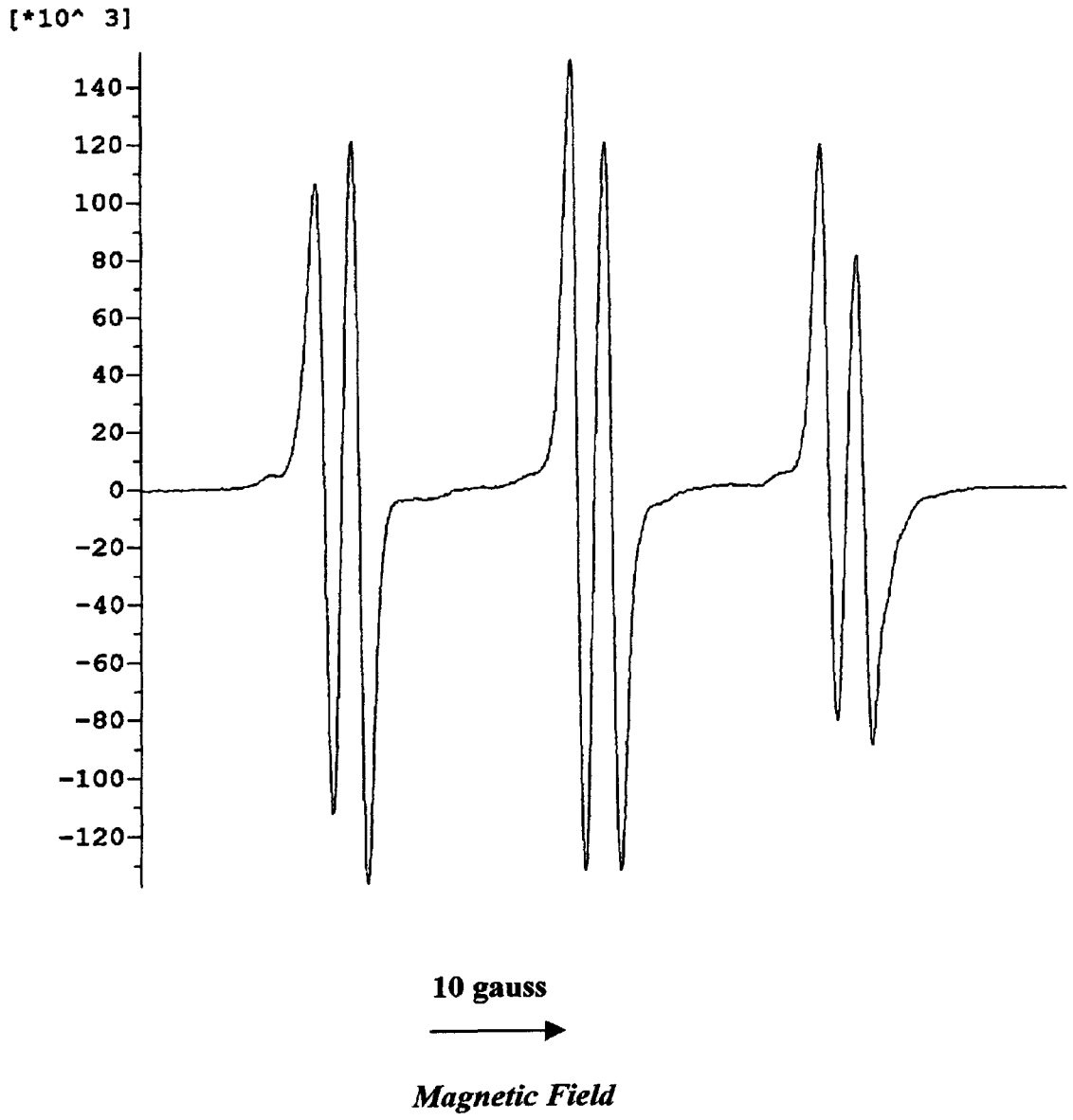


Figure 8.2 shows ESR spectra of α-linolenic acid oxidation. Hyperfine coupling constants for the PBN adducts were measured at a_N, 13.8 gauss; $a\beta_H$, 1.7 gauss. Mean peak amplitude height = 230072 arbitrary units, (n = 6 peaks).

Figure 8.3 – ESR spectra of PBN adduct from arachidonic acid oxidation

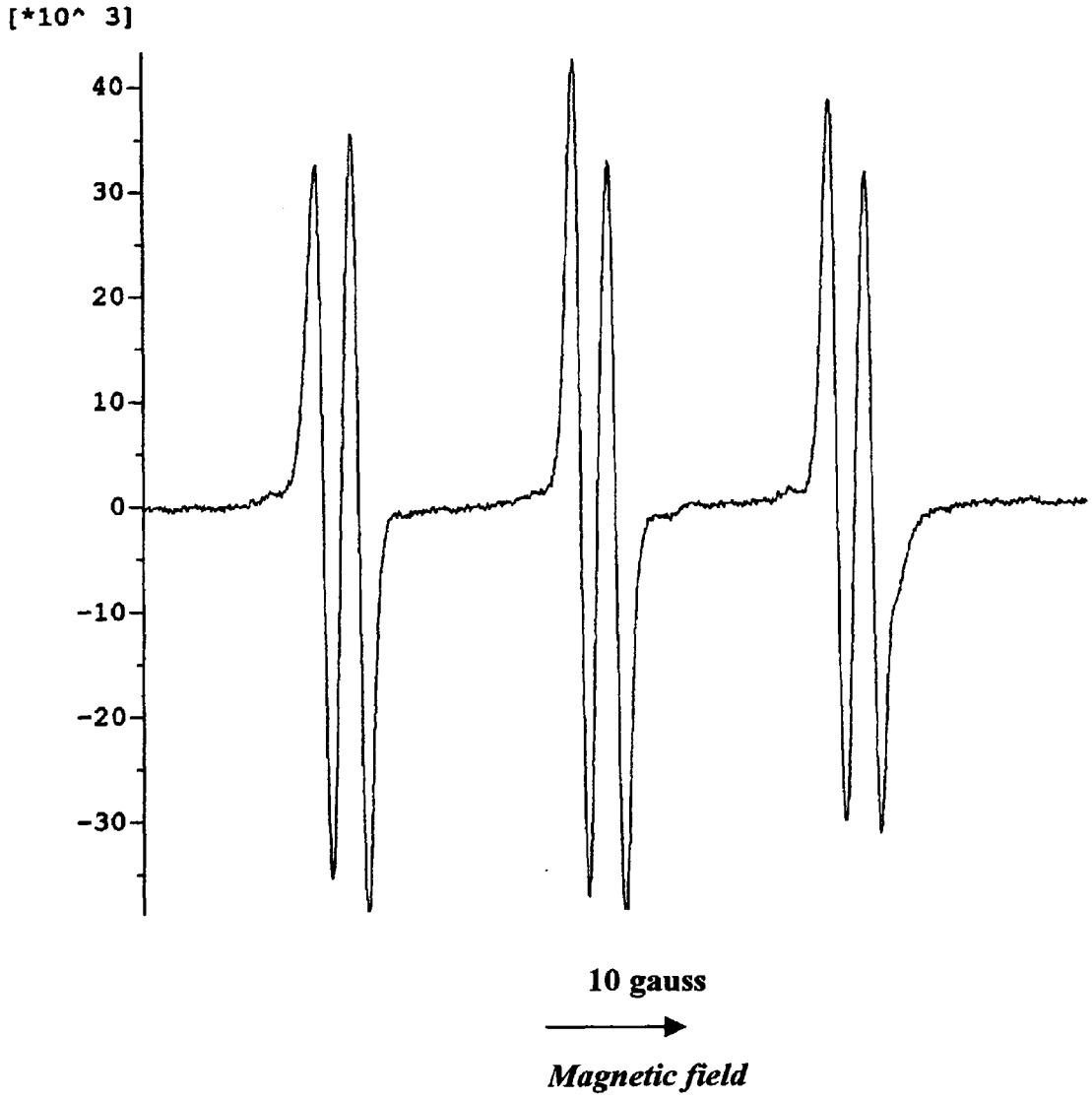


Figure 8.3 shows ESR spectra of arachidonic acid oxidation. Hyperfine coupling constants for the PBN adducts were measured at a_N, 14.0 gauss; $a\beta_H$, 1.9 gauss. Mean peak amplitude height = 70801 arbitrary units, (n = 6 peaks).

Figure 8.4 – ESR spectra of PBN adduct from L-α-phosphatidycholine oxidation

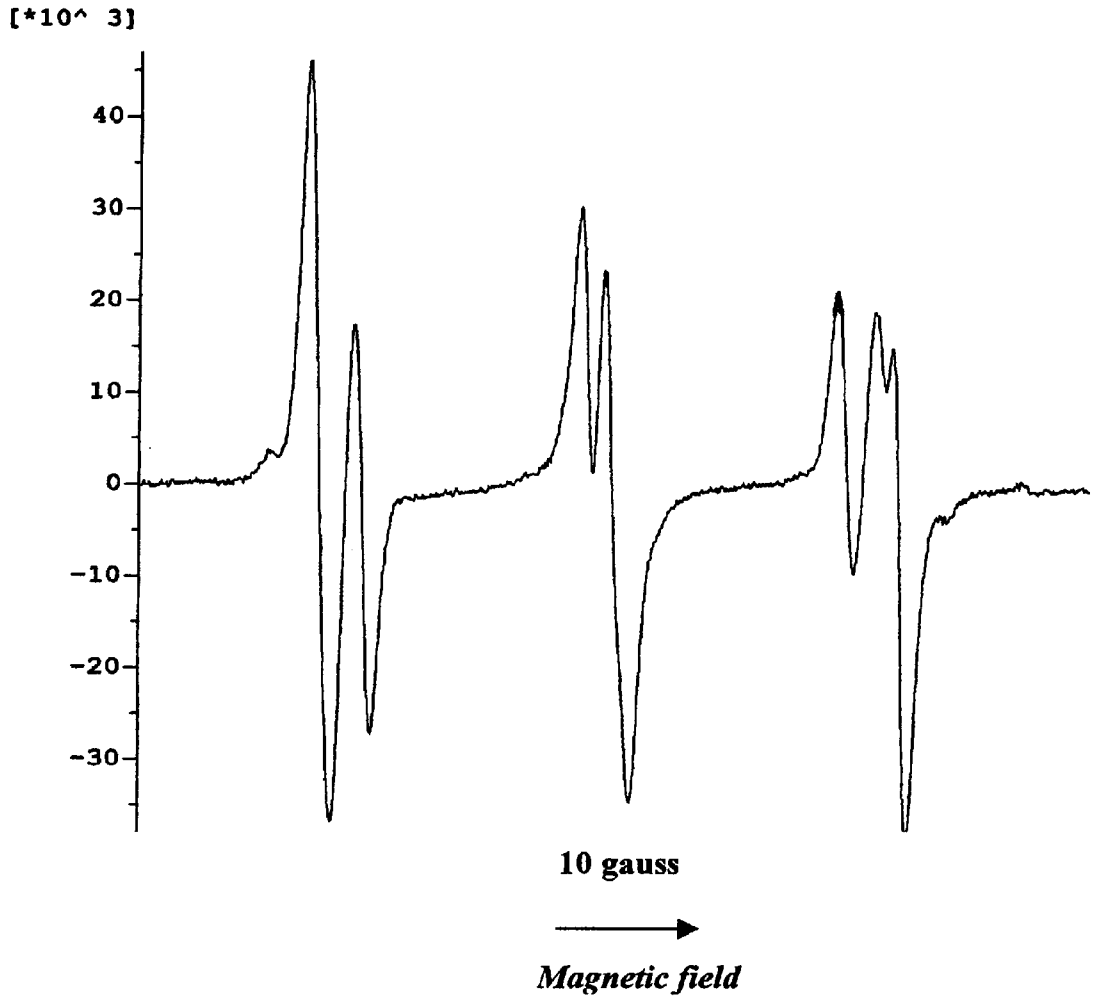
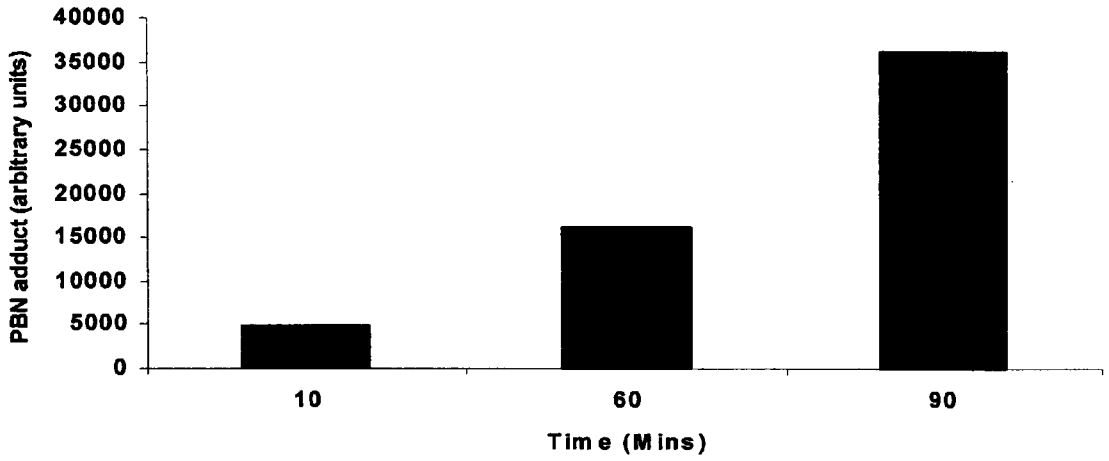


Figure 8.4 shows ESR spectra of L-α-phosphatidycholine oxidation. Hyperfine coupling constants for the PBN adducts were measured at a_N, 14.1 gauss; $a\beta_H$, 2.0 gauss. Mean peak amplitude height = 49620 arbitrary units, (n = 6 peaks). This substrate was prepared by Sigma-Aldrich in 10 mg.ml of chloroform.

Figure 8.5 – PBN adduct of α-linolenic acid oxidation over time



As observed in figure 8.5, there is an increase in free radical concentration as the oxidation of α-linolenic acid increases.

8.3 – DISCUSSION

The primary purpose of these *in vitro* experiments was to attempt to establish whether the free radical species detected in the previous human studies, originate from polyunsaturated fatty acids. All ESR spectra show a triplet of doublets, characteristic of a nitroxide spin adduct, and are similar to the spectra reported in the human studies. The hyperfine coupling constants for linoleic acid, α-linolenic acid, arachidonic acid and L-α-phosphatidycholine were a_N = 13.8 gauss, $a\beta_H$ = 1.8 gauss; a_N = 13.8 gauss, $a\beta_H$ = 1.7 gauss; a_N = 14.0 gauss, $a\beta_H$ = 1.9 gauss; a_N = 14.1 gauss, $a\beta_H$ = 2.0 gauss respectively, and are consistent with the trapping by PBN of secondary oxygen-centred alkoxyl radicals formed via polyunsaturated fatty acid decomposition (Coghlan *et al* 1991, Garlick *et al* 1987).

Other *in vitro* studies report similar coupling constants to those observed in the present chapter. Using Ozone as a PUFA oxidation method, Pryor *et al* (1981) detected PBN adducts from methyl linoleate and suggested that the coupling constants (a_N = 13.7 gauss, $a\beta_H$ = 1.8 gauss) were indicative of an alkoxyl radical. In a recent study, Dikalov

and Mason (2001) using the spin trapping technique has detected alkoxyl free radicals from the oxidation of linoleic, arachidonic and linolenic acid. Both Ashton (1998) and Iwahashi *et al* (1991) report hyperfine coupling constants of a_N = 15.0 gauss, $a\beta_H$ = 2.7 gauss; and a_N = 15.8 gauss, $a\beta_H$ = 2.2 gauss respectively from the *in vitro* oxidation of arachidonic acid, and suggest they resemble the trapping of PBN alkoxyl free radicals. Although the hyperfine constants are slightly higher than those of the present study, Ashton suggests that the ESR spectra of the PBN adducts show evidence of line broadening, which may interfere with spectra measurement. Aside from PBN, Witting *et al* (1999) has detected alkoxyl radicals from the oxidation of L-α-phosphatidylcholine using DMPO. Collectively, these studies support the hypothesis that alkoxyl radicals may be detected from the oxidation of lipid substrates.

Oxygen uptake was shown to increase over the duration of α-linolenic acid oxidation. This finding is in agreement with the work of Mason *et al* (1980), who showed an increase in oxygen consumption with increasing carbon-centred arachidonic acid free radical formation. It is proposed that oxygen is required in increased volume during increased rates of lipid peroxidation. This is supported from data showing an increase in free radical concentration during the various stages α-linolenic acid oxidation.

Moreover, oxygen is fundamental to the progression of the lipid peroxidation chain, as it binds to L$^{\cdot}$ radicals to generate the peroxyl radical (Duthie, 1993), as shown in figure 8.6. It is proposed that this binding/formation would inevitably continue the lipid peroxidation cascade to generate the alkoxyl radical (Halliwell and Gutteridge, 1999) as demonstrated by ESR detection in the present studies.

Figure 8.6 – The role of O_2 in lipid peroxidation

PUFA, polyunsaturated fatty acids; L$^{\cdot}$, lipid radical; O_2, oxygen; ROO$^{\cdot}$, peroxyl radical; ROOH, lipid hydroperoxides; RO$^{\cdot}$, alkoxyl radical.

This data suggests that oxygen is fundamental for both peroxyl and alkoxyl free radical formation. Unfortunately, due to methodology problems oxygen was measured in only one substrate, therefore the oxygen data presented is not representative of all oxidising substrates.

A time course experiment of α-linolenic was performed and monitored by ESR. There was a threefold increase in free radical concentration throughout the period of α-linolenic oxidation. This data clearly shows an accumulation of lipid-derived free radicals over time, and suggests a proportionate increase in oxidative damage. However, a major drawback of this work was the inability to measure the amount of substrate that was oxidised.

All ESR signals were undetectable with the addition of the antioxidant ascorbic acid to the lipid-aqueous mixtures. This is consistent with the known antioxidant properties of ascorbic acid (Ashton *et al*, 1999). However, what is not determined from the current work, is the exact site of ascorbic acid interaction. It is hypothesised that ascorbic acid is scavenging the peroxyl radicals, thus terminating lipid peroxidation and inhibiting the generation of any alkoxyl free radical species. This claim may be supported by work showing a decrease in peroxyl radical detection in the presence of ascorbic acid during methyl linoleate oxidation (Niki *et al*, 1984). Frei *et al* (1989) has also shown that ascorbic acid can protect lipids against oxidative damage that may be induced by peroxyl radicals. Furthermore, Ashton (1998) has shown the complete attenuation of arachidonic acid-derived ESR signals by ascorbic acid intervention.

What is more, Scarpa *et al* (1984) has demonstrated a decrease in the peroxidation rate of soybean L-α-phosphatidylcholine liposomes in the presence of ascorbic acid and α-tocopherol. These authors suggest that the α-tocopherol radical, generated in the phosphatidylcholine bilayer, is regenerated by ascorbic acid, and the scavenging effect of α-tocopherol on lipid peroxidation is maintained only in the presence of ascorbic acid. This is supported by Leung *et al* (1981), who provides evidence for the synergistic effect of ascorbic acid and α-tocopherol as inhibitors of lipid peroxidation in model systems. Moreover, Niki *et al* (1985) has shown in an elegant study that ascorbic acid may scavenge aqueous phase free radicals prior to the oxidation of α-tocopherol.

8.4 – CONCLUSION

These experiments have shown that the PBN trapped free radicals in the human studies are likely to be derived from linoleic acid or/and α-linolenic acid (*as the hyperfine coupling constants are closely matched*), and are identified as secondary alkoxyl radicals. What is not categorically determined from the present research is the contribution of individual membrane lipid to the overall free radical concentration detected in the *in vivo* studies. However, a crude supposition would suggest that α-linolenic is the prominent contributor as the ESR signal amplitude was greater in comparison to linoleic acid oxidation. Furthermore, clear evidence has been provided that ascorbic acid is an effective antioxidant when required to terminate lipid peroxidation and inhibit the generation of alkoxyl radicals.

Chapter 9

Synthesis of Findings

9.0 - Testing of null hypothesis (H_0)

The following section will consider the four hypothesis which were tested by this thesis.

Null hypothesis A – *Direct and indirect indices of free radical-mediated oxidative stress are not affected by normbaric hypoxia.*

HYPOTHESIS ACCEPTED

Despite aerobic exercise increasing the concentration of free radicals and lipid hydroperoxides (*pooled rest vs. exercise,* $P < 0.05$) in systemic blood, no selective differences were observed between the normobaric hypoxic and normobaric normoxic exercise groups (*time x group,* $P > 0.05$). This data provides the basis to reject the alternative hypothesis previous stated as *direct and indirect indices of free radical-mediated oxidative stress are affected by normbaric hypoxia.*

Null hypothesis B - *Exercise-induced oxidative stress levels are not greater in type 1 diabetic patients than in apparently healthy controls.*

HYPOTHESIS ACCEPTED

Oxidative stress was not selectively different (*time x group,* $P > 0.05$) between the diabetic and control groups following exhaustive exercise. However, type 1 diabetic patients had a higher systemic concentration of free radicals and lipid hydroperoxides (*diabetic vs. control,* $P < 0.05$). In addition, exhaustive exercise increased free radical and lipid hydroperoxide concentration (*pooled rest vs. exercise,* $P < 0.05$). This data provides the basis to reject the alternative hypothesis previous stated as *exercise-induced oxidative stress levels are greater in type 1 diabetic patients than in apparently healthy controls.*

Null hypothesis C - *Ascorbic acid supplementation has no effect on oxidative stress levels in type 1 diabetic patients.*

HYPOTHESIS ACCEPTED

Ascorbic acid supplementation did not selectively decrease free radical production in type 1 diabetic patients (*time x group x treatment,* $P > 0.05$). However, ascorbic acid supplementation decreased overall oxidative stress levels (*ascorbic acid vs. placebo,* $P < 0.05$) and following exhaustive exercise (*time x treatment,* $P < 0.05$). This data provides the basis to reject the alternative hypothesis previous stated as *ascorbic acid supplementation affects oxidative stress levels in type 1 diabetic patients.*

Null hypothesis D - *The detected free radical species do not originate from polyunsaturated fatty acids.*

HYPOTHESIS REJECTED

Free radical species were detected from the oxidation of linoleic acid, α-linolenic acid, arachidonic acid and L-α-phosphatidycholine. This data provides the basis to accept the alternative hypothesis previous stated as *the detected free radical species do originate from polyunsaturated fatty acids.*

9.1 – General discussion

The main objectives of the general discussion are to: (1) integrate and summarise the research findings of all studies, and consider the biochemical implications of exercise and oxidative stress in health and disease; and (2) provide direction for future research relating to exercise and oxidative stress in health and disease.

9.1.1 – Integration and summary of research findings

Free radicals are largely recognised as harmful molecules capable of damaging target tissue (see Chapter 2). However the unstable nature of free radicals precludes direct analysis, and hence investigators have relied mainly on the metabolic by-products of lipid peroxidation to quantify the extent of oxidative damage induced by physical exercise and pathology (see Chapter 2). Electron Spin Resonance (ESR) spectroscopy is the most direct, sensitive and specific method available of detecting free radical species in humans. Recently, ESR has been used successfully to detect radical species in venous blood following exhaustive exercise (Ashton *et al*, 1998) and in type 2 diabetes (Anderson *et al*, 2001). The present research was conducted, using ESR spectroscopy in conjunction with the spin trap α-phenyl-*tert*-butylnitrone (PBN), in order to elucidate the possibility that (1) aerobic exercise performed in normbaric hypoxia may generate free radical species, (2) type 1 diabetic patients are more susceptible to exercise-induced oxidative stress and (3) ascorbic acid may attenuate oxidative stress in type 1 diabetic patients.

Exercise and oxidative stress

Studies 1 and 2 demonstrated a significant increase in free radicals following a chronic bout of aerobic exercise and an acute bout of exhaustive exercise respectively ($P < 0.05$). This is evidenced by increases in the concentration of the PBN adduct in the systemic circulation of human volunteers. This free radical increase was associated with an increase in oxygen consumption in both studies, implicating the mitochondria as a potential source of primary oxygen-centred free radical species during exercise. However, the possibility that other pathways such as activated leukocytes may contribute towards an exercise-induced increase in free radical production cannot be

excluded. This claim is supported by an increase in both leukocytes and neutrophils post-exercise in study 1.

The simultaneous rise in free radical production during both exercise challenges was similar, as the concentration of free radicals increased in study 1 by 40.9% and in study 2 by 36%. This would suggest that free radical accumulation in the systemic circulation is neither totally dependent on exercise duration or intensity, but perhaps on an increase in whole body oxygen uptake *per se*. Although both values were within critical difference levels, the possibility that these findings may have some adverse clinical relevance cannot be discarded, since exercise, particularly moderate aerobic exercise, is widely recommended for the general population.

All ESR work in studies 1, 2 and 3 observed hyperfine coupling constants similar to each other (*i.e.* a_N = 13.6 gauss, $a\beta_H$ 1.7 gauss), suggesting that the free radical species detected are lipid-derived secondary oxygen-centred alkoxyl radicals and are similar between studies. Attempts were made to determine the origin of the free radicals observed in these human studies. Similar hyperfine coupling constants were observed from the auto-oxidation of polyunsaturated fatty acids (PUFA), particularly linoleic and α-linolenic acid (see Chapter 8). These findings confirm that the free radicals trapped by PBN in studies 1, 2 and 3 are lipid-derived, possibly from the peroxidation of *in vivo* membrane PUFA by primary oxygen-centred free radicals. The possibility that the species are carbon-centred which may be formed via β-scission of alkoxyl radicals cannot be excluded when dealing with such a complex biochemical system (*Personal communication, Dr T Ashton*).

The leading exercise and free radical hypothesis surrounding this work includes an increase in whole body oxygen consumption and enhanced electron leakage at various levels of the mitochondrial electron transport chain. This may result in the generation of primary free radicals which can attack PUFA's yielding lipid hydroperoxides (LH) and free radicals such as alkoxyl. Studies 1 and 2 observed a significant increase in lipid hydroperoxide concentration in both exercise challenges, confirming the origin of the free radicals species detected and suggesting that physical exercise of different duration and intensity may be associated with cellular membrane damage. This claim is

further supported by data from study 1 showing an elevation in serum creatine phosphokinase (CPK) from pre- to post-exercise. Collectively these data provide evidence that exercise can result in cell membrane damage and increased permeability, possibly due to an increase in free radical production during exercise (*figure 9.0 overleaf*).

Despite higher $\dot{V}O_{2\,max}$ values in comparison to study 2 and an increase in oxygen uptake from pre- to post-exercise ($P < 0.05$), there were no significant changes in free radical or lipid hydroperoxide concentration in study 3 as a function of exhaustive exercise. One explanation of this may be that subject numbers were limited (or alternatively due to ascorbic acid supplementation), thus decreasing the chances of detecting a significant increase. Whilst prospective power calculations were performed for studies 1 and 2, it was difficult to estimate the required subject number needed to gain statistical significance in study 3 given the complex study design.

Contrary to the alternative hypothesis of studies 1 and 2 (see Chapter 1), there were no selective differences in any oxidative stress parameter post-exercise between the normoxic *vs.* hypoxic groups, or between the diabetic *vs.* control group respectively. As the power of the test was calculated for both studies, it may be assumed that the issue of limited subject numbers can be ruled out. However, retrospective power calculations observed throughout this thesis state differently, and it is suggested that this highlights a potential weakness in calculating statistical power prospectively. Thus conclusions drawn in the present thesis may be treated with a certain degree of caution, whilst it is hoped that future work may add to the findings included in this thesis.

Study 1 has shown that prolonged exercise performed at 55% $\dot{V}O_{2\,peak}$ in hypoxia does not selectively increase oxidative stress when compared to normoxic exercise of the same intensity. However, as evident in study 1 (see Chapter 5), the relative contribution by hypoxia to exercise-induced free radical generation was greater in comparison to the normoxic trial. It is proposed that exercising in hypoxia is biochemically more demanding than exercising in normoxia, and the actual percentage of exercise intensity performed in particularly the absolute hypoxic trial is probably higher than 55% of normal $\dot{V}O_{2\,peak}$. This is in line with the previous unpublished observations of Ashton

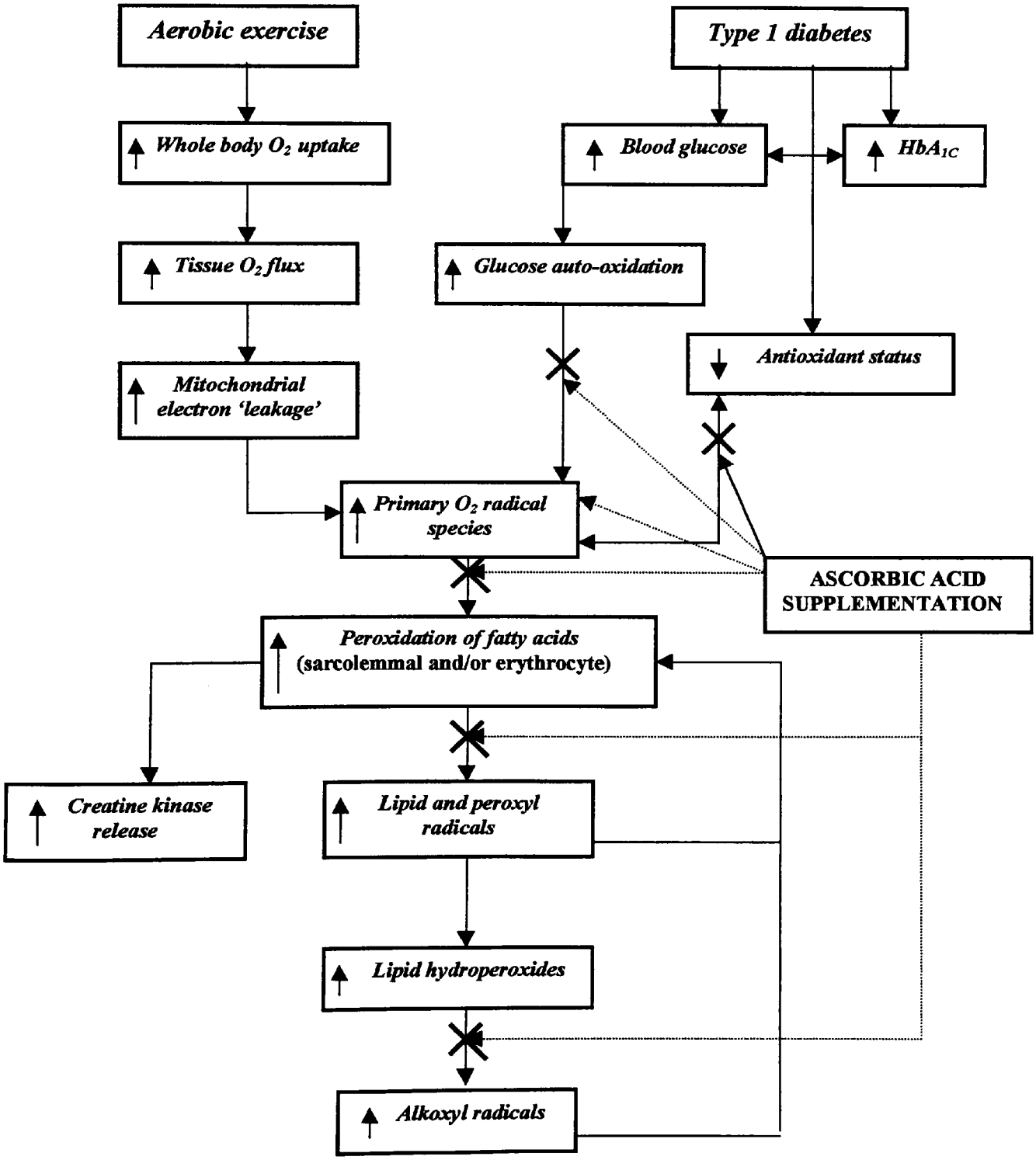
(1998), who has demonstrated using ESR spectroscopy and an *n* of one, the possible existence of an exercise-induced free radical '*threshold*' corresponding to approximately 74% of $\dot{V}O_{2\,max}$. It is pertinent to point out that a free radical threshold may also be regulated by antioxidant status in addition to oxygen uptake. It is further stated that this free radical threshold may have important clinical relevance, as exercising below the identified threshold would allow improved cardiovascular benefits without the potentially detrimental effects associated with elevated free radical production (Ashton, 1998).

Despite the diabetic group having a lower power output and a lesser exercise time to exhaustion, there was no difference in oxidative stress parameters between groups (see Chapter 6). However, the relative increase from pre- to post-exercise for both free radical and lipid hydroperoxide concentrations was greater in the control group (control group, PBN adduct = 66.8%, and LH = 24.7%; diabetic group, PBN adduct = 20% and LH 14.4%). In contrast the absolute free radical and lipid hydroperoxide concentration pre- and post-exercise was higher in the diabetic group (see Chapter 5), although not identified as significantly different from the control group due to the robust statistical approach in data analysis (*i.e* ANOVA). This data is in contrast to the previous work of Laaksonen *et al* (1996), where they have shown a higher absolute oxidative stress at pre- ($P < 0.05$) and at post-exercise ($P < 0.05$) in nine young type 1 diabetic patients in comparison to thirteen control subjects. As these authors used repeated *t*-tests in data analysis, it is possible that a type 1 error occurred (*rejected null hypothesis, when the null hypothesis is true*) (Altman, 1991). However in view of these findings by Laaksonen and colleagues the data in study 2 was also analysed using paired and independent sample *t*-tests, and following this approach the pre-exercise free radical and lipid hydroperoxide data only, of study 2 ($P < 0.05$) matched that of the TBARS data in the study by Laaksonen *et al* (1996). Thus the data in the present thesis provides evidence that diabetic patients are not at a greater risk of oxidative stress whilst exercising.

In the present thesis, there was no change in malondialdehyde (MDA) concentration as a function of any type of exercise challenge (see Chapters 5, 6 and 7). This may be due to (1) the MDA specific assay technique employed in the present research (*i.e.* HPLC), (2) the time point at which MDA was examined in the venous circulation, thus the lack

of detection due to a decreased appearance rate or increased clearance and (3) the increased scavenging of alkoxyl as evident by the decrease in α-tocopherol, lycopene, magnesium and retinol post-exercise.

Figure 9.0 – Hypothetical free radical chain of events and the effect of ascorbic acid supplementation in exercise and in type 1 diabetes.



Study 3 has shown that an increase in free radical concentration following exercise may be attenuated by ascorbic acid supplementation. However, since ascorbic acid affected only the PBN adduct in this manner, it is proposed that the effects of ascorbic acid ingestion on exercise-induced oxidative stress warrants further investigation.

Type 1 diabetes, oxidative stress and ascorbic acid supplementation

Studies 2 and 3 have shown that type 1 diabetic patients have significantly higher venous concentrations of free radicals ($P < 0.05$) and lipid hydroperoxides ($P < 0.05$). This increase in oxidative stress in both studies was associated with an increase in systemic blood glucose concentration and measures of glycemic control (*i.e* HbA_{1c}), which may suggest an increased rate of glucose auto-oxidation as the dominant source of primary oxygen-centred free radical species in the present thesis (*figure 9.0*). The oxidative stress in this group occurred in the presence of a decreased concentration of plasma retinol and lycopene. However it is unknown from the present research whether this antioxidant decrease is a cause or consequence of oxidative stress. In contrast α-tocopherol concentration in the diabetic group was higher in both studies 2 and 3, thus suggesting an inability of this particular antioxidant to combat the generation of aqueous free radical species and oxidative stress.

The significance of this oxidative stress in the pathology of diabetes mellitus is largely unknown (Halliwell and Gutteridge, 1999). However it is proposed that an increase in oxidative stress may contribute towards the development of diabetic vascular complications (Giugliano *et al* 1996, Baynes 1991), as primary free radicals are thought to cause LDL oxidation (*the main contributor to atherosclerosis*) (Steinberg *et al*, 1989) and pronounced endothelial dysfunction (Tesfamariam, 1994), while ascorbic acid is known to restore endothelial function (Lang *et al*, 2001).

Due to the diabetic group in study 2 having increased oxidative stress, study 3 was designed to determine the effect of antioxidant supplementation on oxidative stress in diabetes. Study 3 has shown an attenuation by ascorbic acid supplementation of free radicals and lipid hydroperoxides, although this change was not selectively different between groups. This may in part be due to low subject numbers. Retrospective calculation of statistical power (*observed power calculation* = 0.495) suggests that in

order to achieve a three-way interaction effect, other future studies in the area would require approximately double the existing sample size (*e.g.* approximately $n = 26$ in both groups). However, this is assuming that the effect of ascorbic acid supplementation in the pathology of diabetes is different from healthy subjects, whilst there is no evidence at present to suggest that this may be the case.

Ascorbic acid was chosen as the antioxidant of choice in study 3, as plasma α-tocopherol concentration was generally high even in the presence of an oxidative stress in the diabetic group, and perhaps more importantly, due to the ability of ascorbic acid to target and scavenge aqueous free radicals such as those detected by ESR spectroscopy in the previous studies. Thus the results of study 3 support the general claim that ascorbic acid is an outstanding antioxidant in human blood plasma (Frei *et al*, 1989), and it is proposed that it may interact at various levels of the lipid peroxidation cascade in attenuating oxidative stress as shown in figure 9.0.

Although considerable work regarding exercise, oxidative stress and disease has been published, there are still a number of key questions that have been posed by lead investigators in the field that awaiting answering or confirming, these include:

- Are free radicals produced in excess during exercise?
- Does antioxidant supplementation reduce free radical activity during exercise?
- Are all patients with diabetes more prone to oxidative stress, or only those patients with complications?
- Can oxidative stress in diabetes be decreased by antioxidant therapy?
- Is ascorbic acid a beneficial antioxidant in diabetes?

(Jackson 1996, Halliwell and Gutteridge 1999)

The work in this thesis has incorporated what is widely regarded as the most sensitive, specific and direct method of measuring free radical species, and it is suggested that the ESR studies outlined in this thesis would help to provide answers to these important questions.

Although Ashton *et al* (1998, 1999) were the first to directly show that exercise does increase free radical production in human blood and that ascorbic acid supplementation decreases exercise-induced oxidative stress, the work contained in this thesis has confirmed this notion, and therefore allows with a greater degree of confidence to claim that exercise *per se* does generate free radicals and that antioxidant supplementation may be necessary in physical activity.

The evidence would suggest that patients with uncomplicated type 1 diabetes mellitus are more susceptible to oxidative stress than apparently healthy controls, and this may be due to increased systemic glucose auto-oxidation. This statement may be supported by increased levels of free radicals and cell damage as measured by ESR spectroscopy and free radical-mediated lipid peroxidation respectively. Thus this work has included both direct and indirect assays of free radical activity, thus supporting the validity of claims contained throughout this thesis.

Although ascorbic acid supplementation did not categorically decrease oxidative stress levels in the diabetic group, evidence is provided that overall oxidative stress is decreased by antioxidant therapy, which may be important in diabetes. This work therefore recommends that ascorbic acid is a potentially beneficial antioxidant in exercise and diabetes.

As evident from the following section, much work is still required in the domain of exercise and oxidative stress in both health and disease. It is therefore hoped that the work contained in this thesis provides an induction into the area of free radical research in exercise and pathology, and helps to generate future research ideas.

9.1.2 – Future work required in the area

- There is a general need to apply the technique of ESR spectroscopy to various exercise situations. This approach in addition to measuring the by-products of oxidative damage as supporting markers would help confirm whether free radicals are increased or not in human exercise of different types, intensities and duration. Additionally, the '*threshold*' of exercise-induced oxidative stress needs to be determined.

- There is a general need to apply the technique of ESR spectroscopy to various pathological conditions. For example, evidence has indirectly suggested that patients with Chronic Obstructive Pulmonary Disease (Rahman *et al*, 1996) and Crohn's Disease (Wendland *et al*, 2001) are more susceptible to free radical production, however this still awaits direct confirmation.

- There is a general need to determine the effects of ascorbic acid administration on oxidative stress induced by different types of physical exercise and pathology.

- There is a need to determine the exact dose of ascorbic acid required to diminish exercise or pathologically related oxidative stress. *i.e.* a dose-response relationship needs to be determined. As free radicals are important cell signalling molecules at rest, complete attenuation of these species by antioxidant supplementation may be detrimental to normal cellular function, thus there is also a need to identify an '*adequate*' ingestion dose.

- It is recommended that future studies in the area measure a combination of markers from lipid, DNA and protein oxidation in order to determine the true effects of physical exercise and pathology on oxidative stress.

- There is a general need to determine the effects of exhaustive exercise on long term health.

- The exact identification of the free radical species detected by PBN needs to be resolved. This information would help determine the site of antioxidant scavenging within the lipid peroxidation cascade. It is suggested that the use of ESR spectroscopy in combination with Electron Nuclear Double Resonance (ENDOR) spectroscopy may help resolve this issue.

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Appendices

APPENDIX 1 - TYPICAL MALONDIALDEHYDE CHROMATOGRAPH

Analysis Report

Name: s9
Type: Sample
Sample Amount: 0.000
Scale Factor: 0.000
Injection Volume: 80.0 uL

Vial: a09

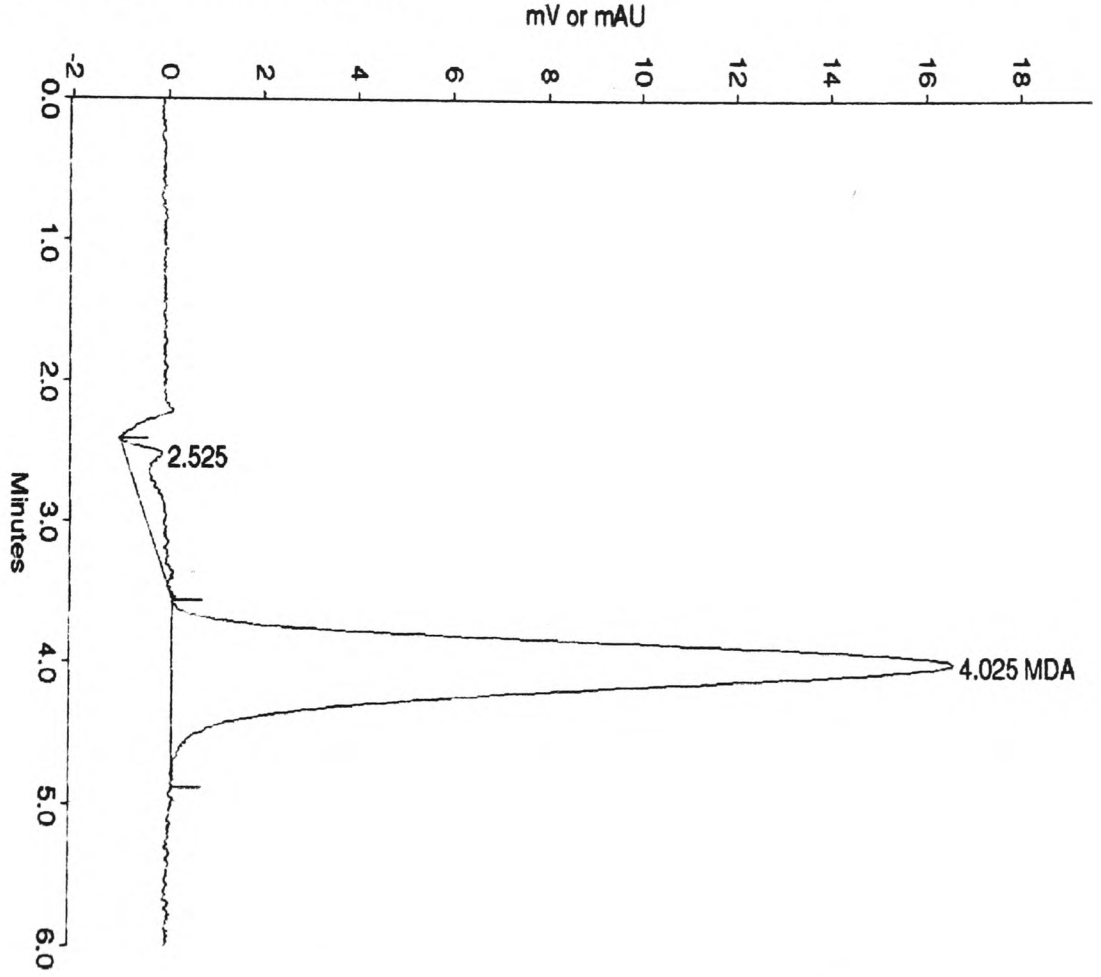
Injection: 1 of 1
Injected On: 09-11-00 11:26:14

Acquisition Log
Column Pressure: N/A
Noise (microAU): 2e+001
Run-Time Messages: None

Column Temperature (C): N/A
Drift (microAU/min): 8e+001

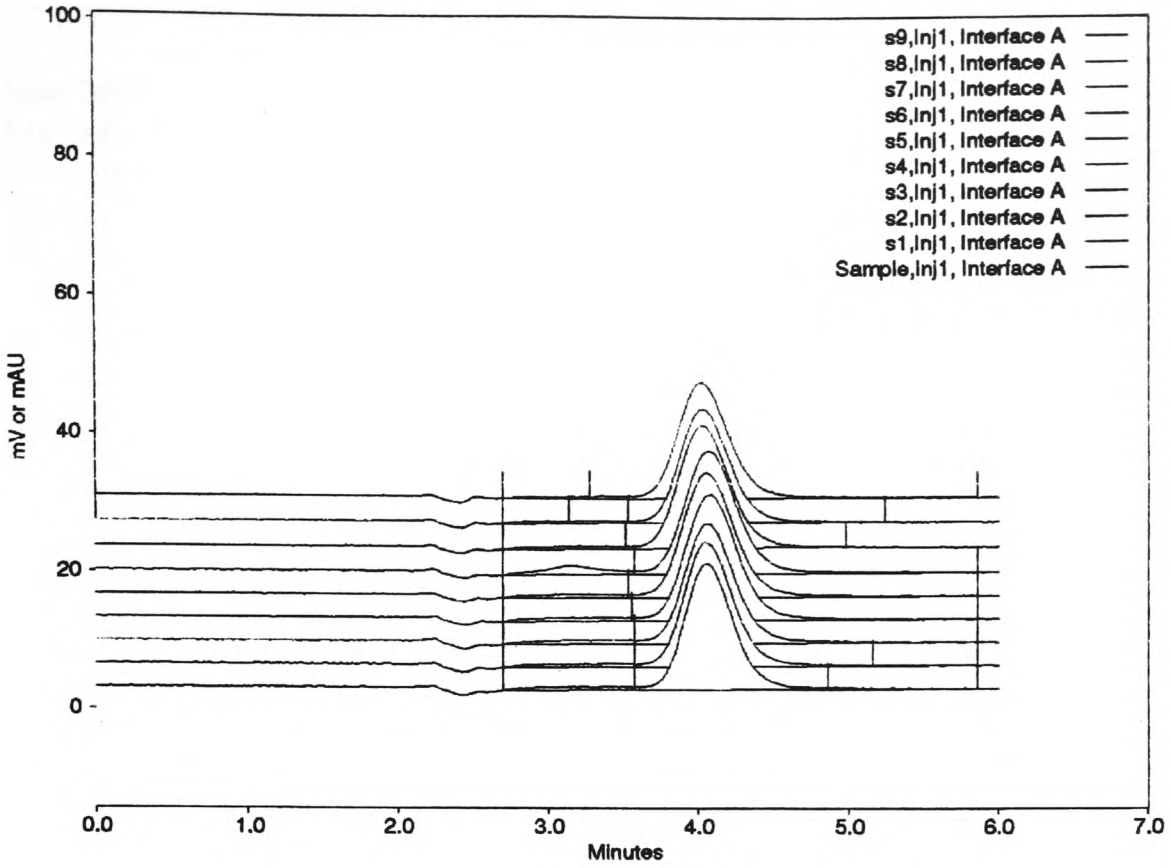
Pump Flow Stability: N/A

Signal 1: Interface A
Calculation Type: Area Percent (Area)



Component	RT(min)	Area	Height	Area%	Peak Type
Unident0001	2.525	21507	759	5.33	Fused
MDA	4.025	382011	16465	94.67	Fused
Totals		403518	17224	100.00	

APPENDIX 2 – MALONDIALDEHYDE COEFFICIENT OF VARIATION



Trend Report

Signal 1: Interface A
Calculation Type: Area Percent (Area)

Acquisition Log

Vial Name	Inj	Col Pressure	Col Temp C	Noise uAU	Drift uAU/min	Pump Flow Stab.	Run-Time Msgs
s1	1	N/A	N/A	20	65	N/A	None
s2	1	N/A	N/A	26	114	N/A	None
s3	1	N/A	N/A	27	-95	N/A	None
s4	1	N/A	N/A	25	51	N/A	None
s5	1	N/A	N/A	23	-23	N/A	None
s6	1	N/A	N/A	27	-42	N/A	None
s7	1	N/A	N/A	26	-5	N/A	None
s8	1	N/A	N/A	25	108	N/A	None
s9	1	N/A	N/A	19	82	N/A	None

Vial Name	Inj	Type	MDA RT(min)	Area	Area%
s1	1	S	4.060	398239	100.000
s2	1	S	4.053	402739	100.000
s3	1	S	4.066	389354	93.788
s4	1	S	4.082	428472	100.000
s5	1	S	4.057	419964	100.000
s6	1	S	4.071	416505	97.585
s7	1	S	4.033	410832	94.952
s8	1	S	4.034	367227	94.070
s9	1	S	4.025	382011	94.670
Mean			4.053	401705	97.229
STD			0.019	19667	2.8371
%RSD			0.476	4.90	2.918

Analysis Report

Name: Calibration 01
Type: Calibration
Internal Standard 1: Tocopherol A
Reference Peak: Tocopherol A
Injection Volume: 80.0 uL

Vial: B01
Level 1 of 1

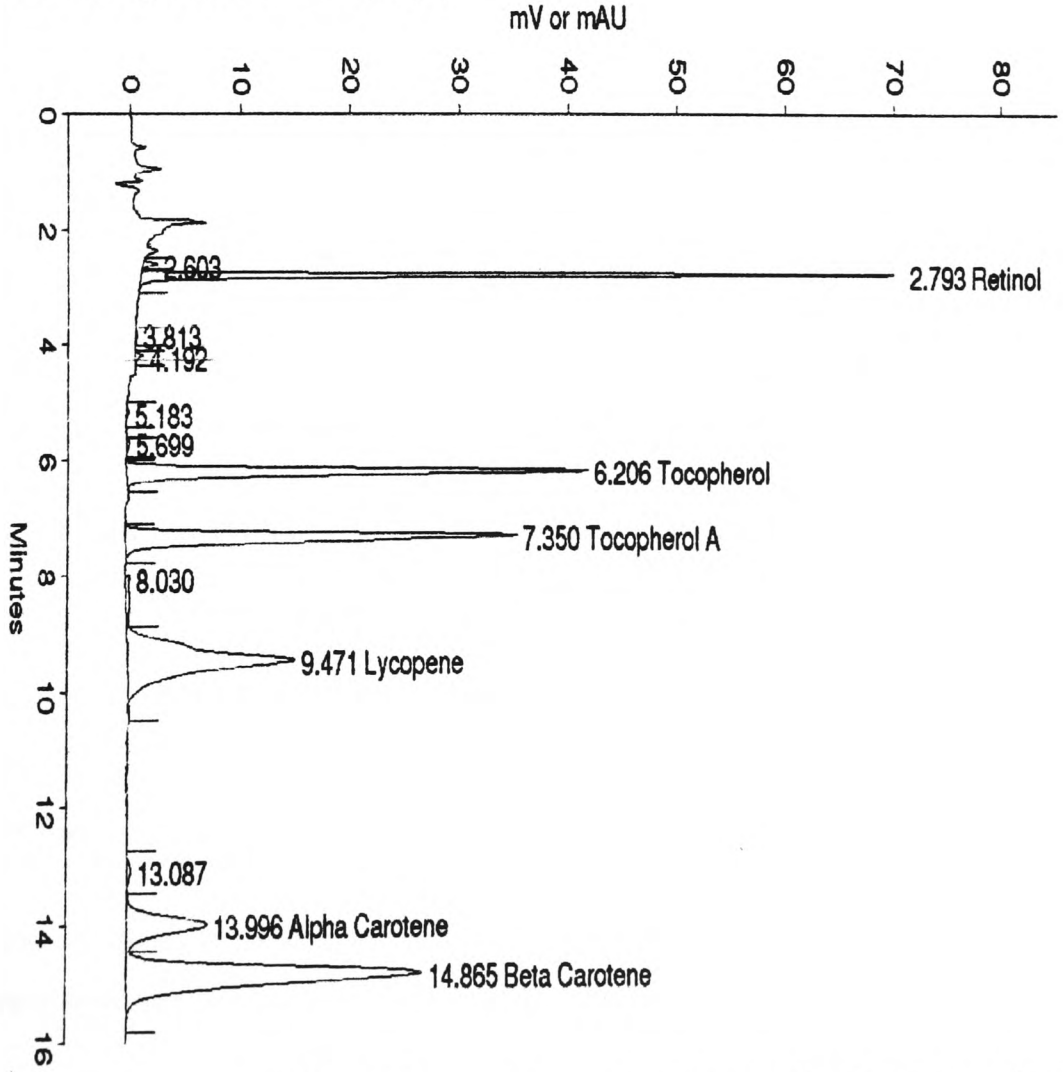
Injection: 1 of 1
Injected On: 05-04-00 19:45:09

Acquisition Log
Column Pressure (PSI): 750
Noise (microAU): 5e+001
Run-Time Messages: None

Column Temperature (C): 35
Drift (microAU/min): 2e+002

Pump Flow Stability: N/A

Signal 1: UV2000 A 325 nm
Calculation Type: Internal Standard (Height)



Component	RT(min)	RRT	Area	Height	uMol/L	Peak Type	RT IS
Unident0001	2.603	0.354	5781	1274	0.0	Fused	0.000
Retinol	2.793	0.380	305815	70012	0.555	Fused	7.350

The aim of the following pilot work was to determine normoxic and hypoxic inspired fractions (F_IO_2) and establish the number of generators needed to regulate the desired inspired fraction.

Percentage (%) and volume of oxygen (O_2) 'pumped' into the chamber from the hypoxic outlet pipe only (flow meter indicating 100%; 9 Psi).

O_2 %	***CO_2 (%)***	***Volume (L)***	***Temperature (°C)***	***Sample time (min)***
14.2	0.07	67.2	22.5	1
14.2	0.03	67.1	22.5	1
14.1	0.03	67.5	22.5	1

Percentage (%) and volume of oxygen (O_2) 'pumped' into the chamber from the hyperoxic outlet pipe only (flow meter indicating 100%; 9 Psi).

O_2 %	***CO_2 (%)***	***Volume (L)***	***Temperature (°C)***	***Sample time (min)***
60.2	0.03	11.0	22.8	1
61.6	0.04	11.5	22.8	1
61.7	0.06	10.8	22.7	1

Percentage (%) and volume of oxygen (O_2) 'pumped' into the chamber from both hypoxic and hyperoxic outlet pipes combined (flow meter indicating 100%; 9 Psi).

O_2 %	***CO_2 (%)***	***Volume (L)***	***Temperature (°C)***	***Sample time (min)***
21.0	0.05	81.4	22.4	1
20.9	0.05	79.9	22.4	1
20.9	0.05	79.1	22.5	1

The combination of both the hypoxic and hyperoxic outlet pipes reflects ambient O_2 concentration, thus confirming the desired procedures for the normoxic *exercise trial.*

4 generators placed inside the chamber

Time (mins)	***F_IO_2 (%)***	***Humidity (%)***	***Temperature (%)***
0	20.7	54	65
15	18.7	54	67
30	16.9	53	71
45	15.5	52	75
60	14.5	52	77

This procedure reduced the F_IO_2 to more than what was desired. It is suggested that hypoxic air was constantly being circulated inside the chamber, thus achieving the observed F_IO_2's. It was decided to reduce the number of generators with the view of increasing the F_IO_2.

3 generators placed inside the chamber

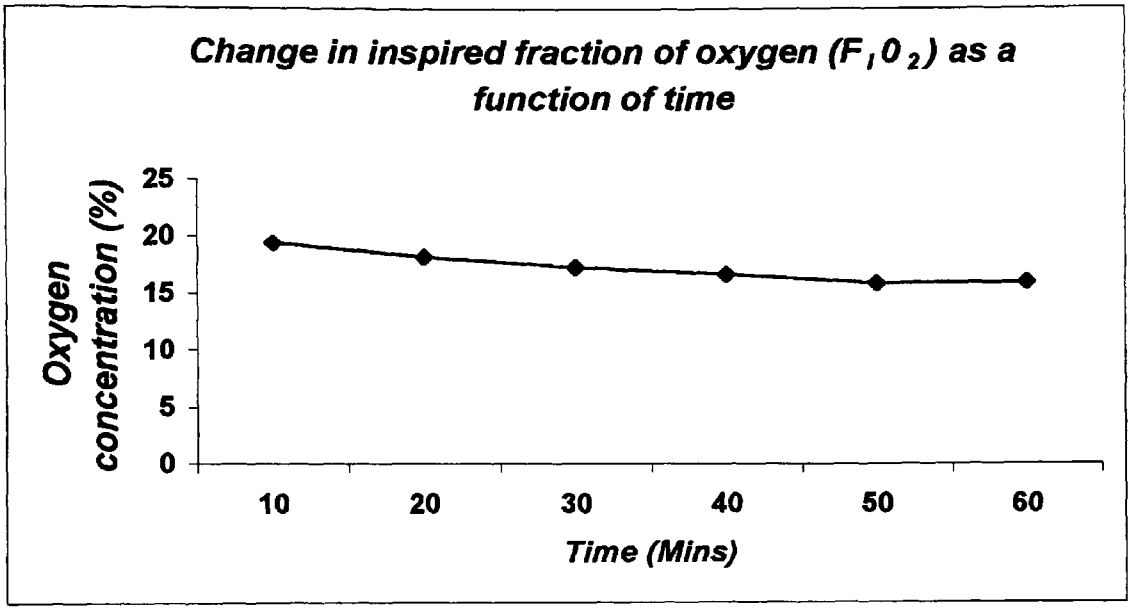
Time (mins)	***F_IO_2 (%)***	***Humidity (%)***	***Temperature (%)***
0	20.6	54	65
15	19.1	54	67
30	17.8	53	71
45	16.6	52	75
60	15.1	52	77

This procedure only slightly increased the F_IO_2, but was still too low in order to conduct the experiment. It was decided to place the 5 generators outside the chamber, thus drawing and filtering ambient air concentrations (*i.e.* 20.93%) into the chamber.

5 generators placed outside the chamber

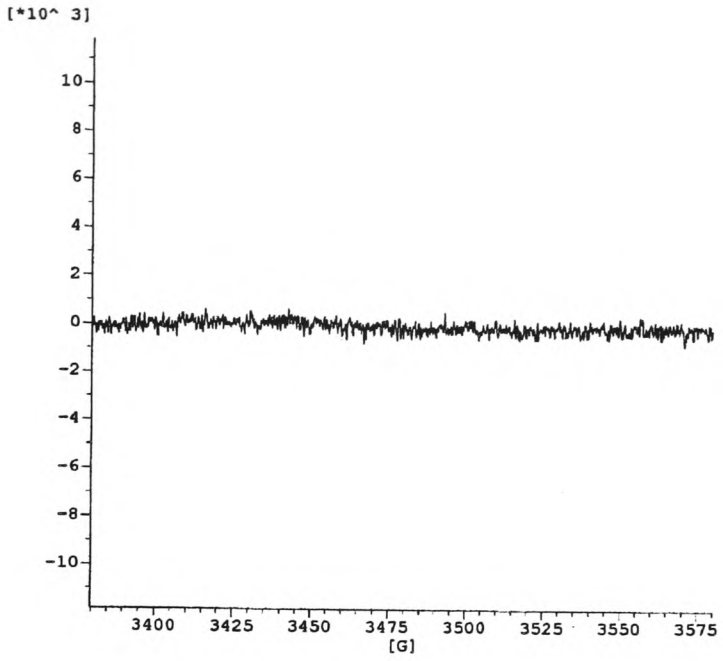
Time (mins)	***F_IO_2 (%)***	***Humidity (%)***	***Temperature (%)***
0	20.9	69	78
10	19.4	69	79
20	18.2	68	79
30	17.3	67	80
40	16.7	66	80
50	15.9	66	81
60	16.0	66	84

The desired F_IO_2 (%) was achieved by placing 5 generators outside the chamber. The figure below shows an exponential decrease in percentage O_2 concentration plotted against time. An equilibrium of 16.0% O_2 was achieved after 60 minutes duration. This served as the procedure to be used in the *hypoxic* exercise trial.



The author would like thank Miss R Morgan for her contribution to pilot work data collection.

APPENDIX 5 – EMPTY BRUKER CAVITY



Parameter List

Operator: Gareth
Resonator: c:\...\tm_9808.cal
Acqu. Date: 14.Sep.2000
# of Scans: 10

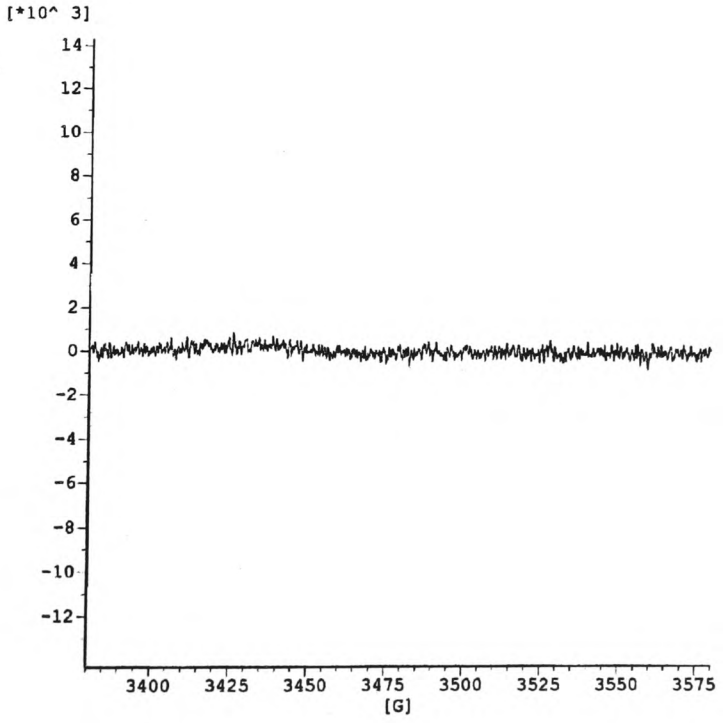
Field
Center Field: 3480.000 G
Sweep Width: 200.000 G
Resolution: 2048 points

Microwave
Frequency: 9.810 GHz
Power: 20.117 mW

Receiver
Receiver Gain: 1.00e+005
Phase: 0.00 deg
Harmonic: 1
Mod. Frequency: 100.00 kHz
Mod. Amplitude: 0.50 G

Signal Channel
Conversion: 40.960 ms
Time Constant: 81.920 ms
Sweep Time: 83.886 s

APPENDIX 6 – BLANK ESR TUBE



Parameter List

Operator: Gareth
Resonator: c:\...\tm_9808.cal
Acqu. Date: 14.Sep.2000
# of Scans: 10

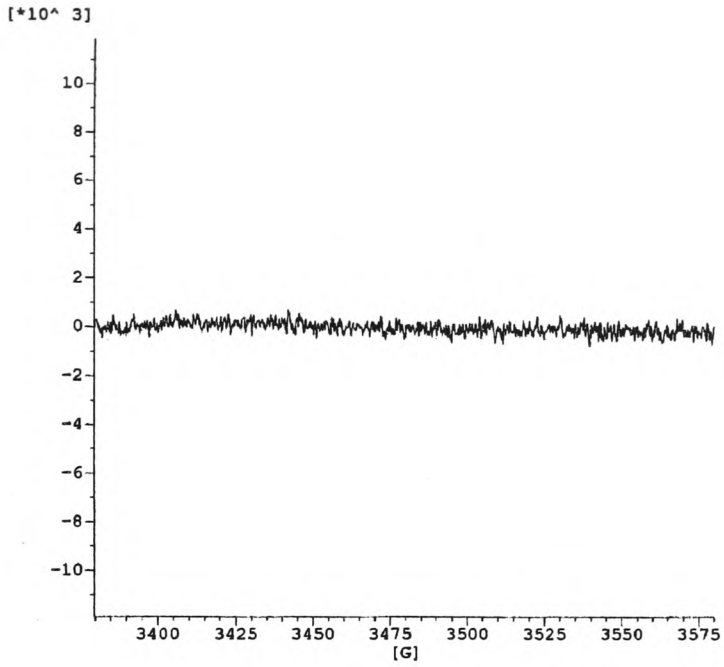
Field
Center Field: 3480.000 G
Sweep Width: 200.000 G
Resolution: 2048 points

Microwave
Frequency: 9.795 GHz
Power: 20.117 mW

Receiver
Receiver Gain: 1.00e+005
Phase: 0.00 deg
Harmonic: 1
Mod. Frequency: 100.00 kHz
Mod. Amplitude: 0.50 G

Signal Channel
Conversion: 40.960 ms
Time Constant: 81.920 ms
Sweep Time: 83.886 s

APPENDIX 7 – DEGASSED TOLUENE



Parameter List

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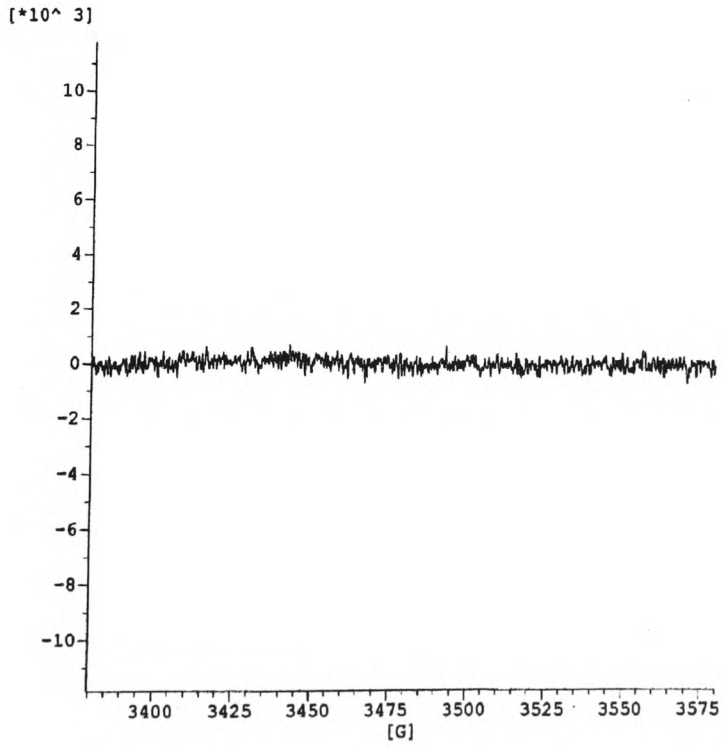
# of Scans:	10
Field	
Center Field:	3480.000 G
Sweep Width:	200.000 G
Resolution:	2048 points
Microwave	
Frequency:	9.795 GHz
Power:	20.117 mW
Receiver	
Receiver Gain:	1.00e+005
Phase:	0.00 deg
Harmonic:	1
Mod. Frequency:	100.00 kHz
Mod. Amplitude:	0.50 G
Signal Channel	
Conversion:	40.960 ms
Time Constant:	81.920 ms
Sweep Time:	83.886 s

APPENDIX 8 – UNTREATED WHOLE BLOOD



Parameter List

Operator:	Gareth
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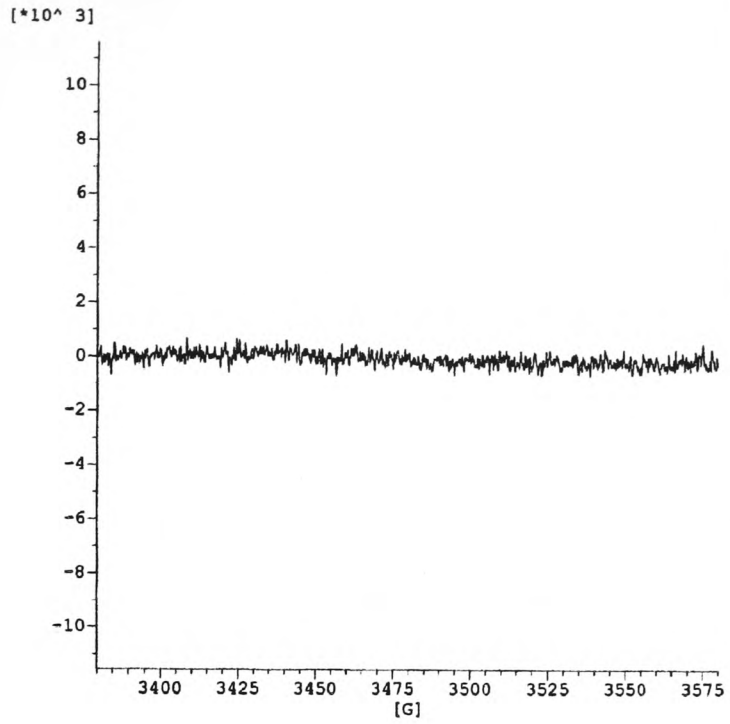
Field	
Center Field:	3480.000 G
Sweep Width:	200.000 G
Resolution:	2048 points
Microwave	
Frequency:	9.810 GHz
Power:	20.117 mW
Receiver	
Receiver Gain:	1.00e+005
Phase:	0.00 deg
Harmonic:	1
Mod. Frequency:	100.00 kHz
Mod. Amplitude:	0.50 G
Signal Channel	
Conversion:	40.960 ms
Time Constant:	81.920 ms
Sweep Time:	83.886 s

APPENDIX 9 – SERUM, NO PBN



Parameter List

Operator:	Gareth
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Acqu. Date:	08.Sep.2000
# of Scans:	10
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Center Field:	3480.000 G
Sweep Width:	200.000 G
Resolution:	2048 points
Microwave	
Frequency:	9.800 GHz
Power:	20.120 mW
Receiver	
Receiver Gain:	1.00e+005
Phase:	0.00 deg
Harmonic:	1
Mod. Frequency:	100.00 kHz
Mod. Amplitude:	0.50 G
Signal Channel	
Conversion:	40.960 ms
Time Constant:	81.920 ms
Sweep Time:	83.886 s

APPENDIX 10 – DPPH SAMPLE